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# HIF-1α in the Heart: Provision of Ischemic Cardioprotection and Remodeling of Nucleotide Metabolism

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# HIF-1α in the Heart: Provision of Ischemic Cardioprotection and Remodeling of Nucleotide Metabolism

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A dissertation

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by

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ABSTRACT

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In our studies we found that stabilized expression of HIF-1α in heart led to better recovery of function and less tissue death after 30 minutes of global ischemia, via mechanisms that preserve the mitochondrial polarization. Our group previously showed that HIF-1α conferred ischemic tolerance by allowing cardiomyocytes to use fumarate as an alternative terminal electron acceptor to sustain anaerobic mitochondrial polarization. The source of fumarate was identified as the purine nucleotide cycle (PNC). Here we discovered that HIF-1α upregulates AMP deaminase 2 (AMPD2), the entry point to the PNC. The combination of glycolysis and the PNC may protect the heart's nucleotide resources. We subsequently examined the effects that HIF-1α exerts on nucleotide metabolism in the ischemic heart. We found that HIF-1α expression reduces adenosine accumulation in the ischemic heart. As ATP is depleted during ischemia, AMP accumulates. Our results suggest that AMP metabolism is shunted towards AMPD2 rather than the adenosine producing 5'-nucleotidase pathway. Subsequently, we treated hearts with the PNC inhibitor hadacidin followed by 30 minutes of global ischemia. Inclusion of hadacidin reduced ATP and adenylate energy charge in the hearts. These findings allow us to propose that activity of the PNC prevents the $F_0F_1$ ATP synthase from consuming glycolytic ATP in order to maintain mitochondrial polarization during ischemia. Thus, the PNC provides ATP sparing

effects and preserves the energy charge in the ischemic heart. The fact that ATP and adenylate energy charge is better preserved during the initial 20 minutes of ischemia in HIF-1α expressing hearts is supportive of our observation that HIF-1α upregulates the PNC. HIF-1α also upregulates adenosine deaminase, which degrades adenosine. The limitation of adenosine accumulation may help HIF-1α expressing hearts avoid toxicity due to chronic adenosine exposure. Finally, we found that HIF-1α induces the expression of the nucleotide salvage enzyme hypoxanthine phosphoribosyl transferase (HPRT). Upon reperfusion HPRT serves to reincorporate the nucleotide degradation product, hypoxanthine, into the adenylate pool and may prevent the production of reactive oxygen species. Collectively, HIF-1α robustly protects the heart from ischemic stress and it upregulates several pathways whose cardioprotective role may extend beyond the remodeling of nucleotide metabolism.

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Last but not least, thanks goes out to my teachers at Wellington Community High School in Florida. Mrs. Cloran, Mrs. Williams, Mr. Walsh, and Mr. Burgio, you guys have truly been the first people to stimulate my interest in math and science. To my AP U.S. history teacher, Mr. Sidway, I will never forget your inspirational quote... "Stick around, you will be surprised at the outcome and how well things will turn out".

# DEDICATION

I dedicate this manuscript to my parents, you have always pushed me to the limit and now your strict guidance is paying off. Thanks! I love you guys! To my sister Jenny, thank you for providing so many years of laughter and joy! To my friends and fellow graduate students in the BMS program, thanks for providing the distractions and the constant reminder that I need a social life too. To Pam Scollard, Vicki Matthew, and Dr. Harold Cates, I would not be standing here today without you.

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# CHAPTER 1

# INTRODUCTION

Diatomic oxygen ($O_2$) has not always been a major component of the earth's atmosphere. For instance, during the early Proterozoic era $O_2$ content was less than $10^{-5}$ times that found in the present day atmosphere (Pavlov and Kasting 2002). During that time photosynthetic organisms released $O_2$ into the atmosphere as a metabolic waste product. However, the $O_2$ released readily reacted with iron and other organic matter (i.e. $O_2$ sinks). Approximately 2.4 billion years ago the $O_2$ sinks became saturated and atmospheric $O_2$ levels began to accumulate to that found in the present day atmosphere. Concurrently, $O_2$ consuming organisms started to evolve.

In regards to the importance of oxygen in sustaining life of aerobic organisms, Semenza has said that "no substance which when withheld causes death faster" (Semenza 1998). This is because $O_2$ is crucial to cellular survival as it is coupled to oxidative phosphorylation in order to produce the cell's energy currency, ATP (Figure 1.1). Mechanistically, electron pairs can enter the electron transport chain at complex I (I, NADH-CoQ reductase) or complex II (II, succinate-CoQ reductase). Electrons proceed to complex III (III, CoQ-cytochrome C reductase) then to complex IV (IV, cytochrome C oxidase) (Figure 1.1). At complexes I, III, and IV, $H^+$ ions are translocated into the intermembrane space as electrons proceed down the redox gradient. A total of 10 or 6 $H^+$ are translocated depending on whether NADH or succinate is used as the initial electron donor. $4H^+$ re-enter the mitochondrial matrix to power the $F_0F_1$ ATP synthase so that production of ATP can occur. Compared to

glycolysis alone, which only generates a net of 2 ATP molecules per glucose, aerobic respiration can generate 30 ATP molecules per glucose and is therefore much more efficient (Rich 2003). Given the importance of $O_2$ for survival, an intricate method of oxygen sensing and ability to adapt in oxygen poor environments exists. This is evident by the ability of humans to survive altitudes as high as 29,000 feet as in the case of Messner and Habeler in their ascend of Mount Everest without oxygen supplementation.

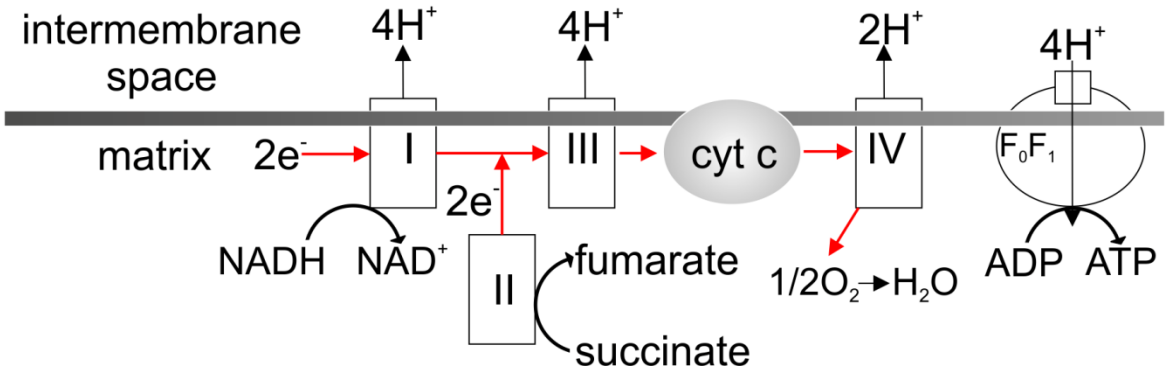


Figure 1.1: *$O_2$ is coupled to the synthesis of ATP via oxidative phosphorylation*. Electrons can enter the electron transport chain at I (NADH-CoQ reductase) or at II (succinate-CoQ reductase). In either case, $2e^-$ are passed onto III (CoQ-cytochrome C reductase) then onto IV (cytochrome C oxidase). The passing of an electron pair down the electron transport chain leads to 10 $H^+$ and 6 $H^+$ that are translocated to the intermembrane space depending on whether NADH or succinate is used as the initial donor, respectively. The translocation of $H^+$ into the intermembrane space sets up an electrochemical gradient, which is then used to power ATP synthesis. Adapted from Lodish et al. (2007).

The cell's adaptive responses to inadequate oxygen supply is mediated by a transcription factor known as hypoxia inducible factor-1 (HIF-1). HIF-1 was identified and named by Semenza and Wang in their effort to find the transcription factor binding to a 50 nucleotide hypoxia inducible enhancer on the human erythropoietin gene (Semenza and Wang 1992). Later work revealed that HIF-1 is composed of a 120 kDa

α subunit (HIF-1α) and a 91-94 kDa β subunit (HIF-1β) (Wang and Semenza 1995). Structurally, HIF-1 belongs to the basic helix-loop-helix PAS family of transcription factors (Wang et al. 1995).

While HIF-1β is constitutively expressed in the nucleus (Jiang et al. 1996), the cellular level of HIF-1α is tightly regulated by $O_2$. Under normoxic conditions a family of prolyl hydroxylase domain containing enzymes (PHDs) hydroxylate proline residues (Bruick and McKnight 2001; Epstein et al. 2001) on HIF-1α's oxygen-dependent degradation (ODD) domain. It has been recognized that these residues are proline 402 and proline 564 (Ivan et al. 2001; Jaakkola et al. 2001; Yu et al. 2001; Chan et al. 2005). Hydroxylation of these proline residues subsequently allows for interaction of von Hippel-Lindau (VHL) tumor suppressor with HIF-1α. Once VHL binds, HIF-1α becomes polyubiquintinated and is subsequently directed towards proteasomal degradation (Figure 1.2). The transcriptional activity of HIF-1α is also controlled by oxygen. During normoxia factor inhibiting HIF (FIH) hydroxylates asparagine residue 803 (Mahon et al. 2001; Hewitson et al. 2002; Lando, Peet, Gorman, et al. 2002). Hydroxylation of this asparagine residue prevents the binding of transcriptional coactivators CBP/p300 to HIF-1α's c-terminal transactivation domain (Lando, Peet, Whelan, et al. 2002). Upon stabilization HIF-1α translocates into the nucleus and dimerizes with the β subunit (Jiang et al. 1996; Chilov et al. 1999) where transcription of genes containing the core 5'-RCGTG-3' hypoxic response element occurs (Semenza et al. 1996) (Figure 1.3).

Hypoxia inducible factor-1 has been shown to elicit the upregulation of a vast array of genes. An important adaptation mediated by HIF-1 is ATP production in the absence of oxidative phosphorylation via glycolysis. As such, the core hypoxic response element has been found in genes encoding various enzymes of the glycolytic pathway (Semenza et al. 1996). HIF-1 also stimulates the expression of glucose transporters in order to increase cellular glucose uptake (Hayashi et al. 2004; Baumann et al. 2007). Hypoxia inducible factor overexpression is a common theme observed in cancer and is associated with VHL deficiency in renal cell carcinoma (Maxwell et al. 1999; Zhong et al. 1999). Therefore, it was not surprising that German physiologist Otto Warburg observed that cancer cells readily produce ATP through glycolysis rather than oxidative phosphorylation even in the presence of oxygen. This phenomenon was rightfully named the "Warburg effect" (Warburg 1956). Semenza has recently found that HIF-1α drives the Warburg effect in cancer (Semenza 2007). Besides the induction of glycolysis HIF-1 also upregulates genes that promote oxygen delivery to the cell. As mentioned previously, HIF-1 is responsible for inducing the expression of erythropoietin, which stimulates red blood cell synthesis and an increase in hematocrit (Semenza and Wang 1992). Furthermore, HIF-1 increases the expression of vascular endothelial growth factor (VEGF) to promote angiogenesis (Liu et al. 1995; Carmeliet et al. 1998; Ryan et al. 1998).

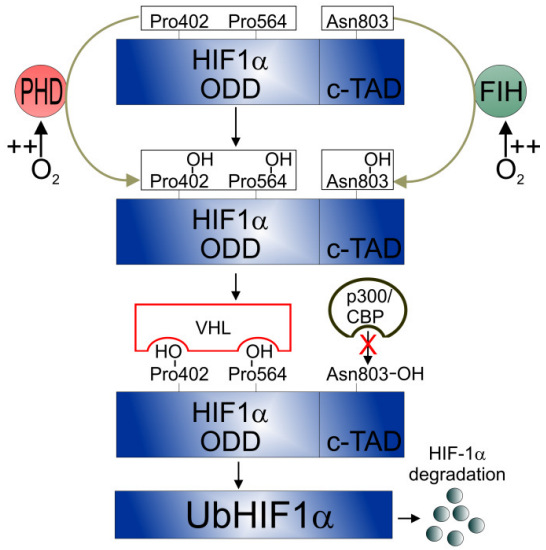


Figure 1.2: *HIF-1α protein level and activity is tightly regulated by oxygen.* Hydroxylation of proline (Pro) residues 402 and 564 by prolyl hydroxylase (PHD) allows von Hippel-Lindau (VHL) factor to recognize and bind to HIF-1α's oxygen dependent degradation domain (ODD). Subsequently HIF-1α is ubiquitinated and directed towards proteasomal degradation. Under normoxic conditions HIF-1α transcriptional activity is also suppressed. Factor inhibiting HIF (FIH) hydroxylates asparagine (Asn) residue 803, thus preventing the binding of transcriptional co-activators CBP/p300 at HIF-1α's c-terminal transactivation domain (cTAD). Adapted and modified from Semenza (2004).

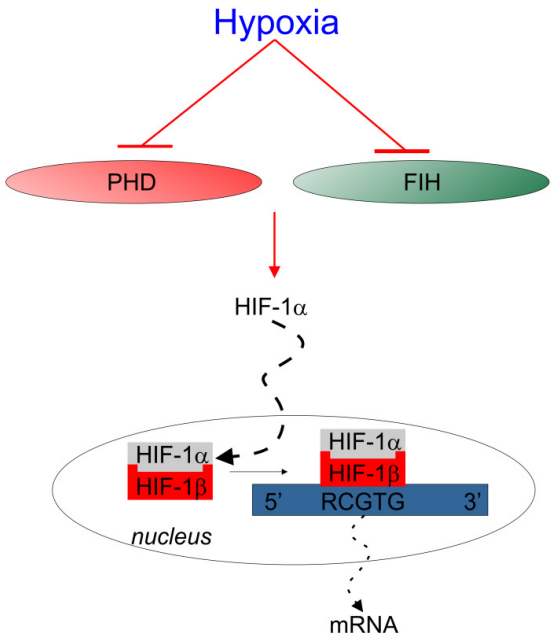


Figure 1.3: *Decline in $O_2$ during hypoxia suppresses the activity of PHDs and FIH, thus stabilizing and activating HIF-1α.* $O_2$ is a cofactor that is needed for the enzymatic activity of

PHDs and FIH. When $O_2$ level declines, PHDs and FIH activity is abrogated thus stabilizing and activating HIF-1α. HIF-1α translocates into the nucleus, dimerizes with HIF-1β to initiate the transcription of genes containing the core 5'-RCGTG-3' hypoxia response element (HRE). Adapted and modified from Semenza (2004).

$O_2$ delivery to the heart tissue is diminished during ischemia. It is now recognized that HIF-1α may play a central role in cardioprotection during ischemic stress. Ischemic preconditioning is a phenomenon whereby exposure of hearts to short sublethal durations of ischemia-reperfusion is able to provide protection from further damage upon exposure to subsequent longer and lethal episodes of ischemic stress. Following a preconditioning protocol consisting of 4 rounds of 5 minutes of ischemia/5 minutes of reperfusion an increase in HIF-1α protein levels was observed (Eckle et al. 2008). In mouse models with a null allele of the HIF-1α locus a loss of preconditioning protection in heart was noted (Cai et al. 2003; Cai et al. 2008). Also, repression of HIF-1α via siRNA was found to attenuate ischemic preconditioning protection (Eckle et al. 2008). Ockaili et al. and Poynter et al. demonstrated that the use of pharmacologic inhibitors of prolyl hydroxylase to induce HIF-1α also confers cardioprotection (Ockaili et al. 2005; Poynter et al.). Similarly, administration of siRNA which silences prolyl hydroxylase or knockout of prolyl hydroxylases to stabilize HIF-1α have proven to be cardioprotective (Natarajan et al. 2006; Hyvarinen et al.). A drawback to studies using PHD inhibitors or knockout is that prolyl hydroxylases mediates pathways in addition to HIF-1α (Cummins et al. 2006; Elvidge et al. 2006). As a result ischemic cardioprotection provided by PHD inhibition cannot be solely attributed to HIF-1α expression. Nevertheless, recent findings strongly indicate a prominent role for HIF-1α in cardioprotection during ischemia.

As previously mentioned, HIF-1α is degraded in the presence of oxygen. In order to assess the direct effects that HIF-1α expression exerts on the heart, we used a mouse model containing a cardiac-specific and doxycycline regulated HIF-1α transgene that is mutated to be stable and active in the presence of oxygen, thus circumventing the oxygen mediated degradation and suppression of HIF-1α. Using this mouse model we sought to: 1) establish the cardioprotective role of HIF-1α; 2) identify HIF-1α mediated mechanisms that provide ischemic cardioprotection; 3) evaluate the metabolic changes in the heart as a result of HIF-1α expression.

# CHAPTER 2

## HIF-1α IN THE HEART: PROTECTIVE MECHANISMS

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## SUMMARY

Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that directs many of the cellular responses to hypoxia. In these studies, we have used a mouse model containing a cardiac-specific, oxygen-stabilized, doxycycline (Dox-off) regulated HIF-1α transgene to probe the role of HIF-1α in cardioprotection. Hearts used in these studies were derived from wildtype (WT) mice, as well as those maintained on doxycycline to suppress the HIF-1α transgene (Non-I) or denied doxycycline to express the HIF-1α transgene for 2 days (2D) or 6 days (6D). Whereas HIF-1α protein is undetectable in WT mouse hearts, it is present in heart tissue of Non-I transgenic mice, presumably because of leakiness of the promoter construct. In mice denied doxycycline for 2 or 6 days, HIF-1α is overexpressed to a much greater extent than Non-I or WT animals, as expected. WT and HIF-1α expressing hearts (Non-I, 2D and 6D induced) were subjected to 30 minutes of ischemia, and functional recovery was measured upon reperfusion. Recovery of pre-ischemic left ventricular developed pressure was 14% for WT, 67% for Non-I hearts, 64% for 2D, and 62% for 6D hearts. 6D hearts have increased pre-ischemic glycogen reserves, higher glycogen synthase protein levels, and significantly higher lactic acid release during ischemia. 6D HIF hearts were also better able to maintain ATP levels during ischemia compared with WT and Non-I hearts. Interestingly, Non-I hearts showed no significant increase in glycogen reserves,

glycolytic flux, or greater ATP preservation during ischemia and yet were protected to a similar extent as the 6D hearts. Finally, the mitochondrial membrane potential of isolated adult myocytes was monitored during anoxia or treatments with cyanide and 2-deoxyglucose. HIF-1α expression was shown to protect mitochondrial polarization during both stress treatments. Taken together, these data indicate that, while HIF-1α expression in heart does induce increases in compensatory glycolytic capacity, these changes are not necessarily required for cardioprotection, at least in this model of ischemic stress.

## INTRODUCTION

Hypoxia-inducible factor-1α (HIF-1α) is a master regulatory transcription factor that directs the cellular response to hypoxia. When in vivo oxygen tension is sufficient, HIF-1α is hydroxylated at proline residues 402 and 564 by a family of prolyl hydroxylase domain-containing proteins (PHDs1–3) (2, 27). Hydroxylation of HIF-1α results in its recognition by von Hippel-Lindau factor and its subsequent degradation via proteasomal pathways. Hydroxylation of asparagine residue 803 (Asn803) by factor-inhibiting HIF provides an additional regulatory mechanism for HIF-1α (10, 13). The hydroxylation of Asn803 blocks the binding of coactivators CBP/p300 to the C-terminal activating domain of HIF-1α, thereby limiting its transcriptional activity. Thus, HIF-1α is both stabilized and its transcription activity is increased through these mechanisms when oxygen tension drops. The present studies are designed to establish if HIF-1α is sufficient to confer protection to the heart against ischemia-reperfusion injury and to begin to explore the mechanisms through which this protection is afforded. In pursuit of these aims, we used a transgenic mouse model containing a HIF-1α cDNA with alanine substitutions at

Pro402, Pro564, and Asn803 as described by Bekeredjian et al. (1). These substitutions result in a HIF-1α protein, termed HIF-1α-PPN, that is stable and displays full transcriptional activity in normoxic conditions. A tetracycline-regulated construct was used to obtain regulation of transgene expression. In the presence of doxycycline, transgene transcriptional expression is suppressed and induced when doxycycline is excluded from the diet. The expression of the tetracycline transactivator protein is driven by a cardiac-specific myosin heavy chain promoter, thus limiting HIF-1α transgene expression to the cardiac myocytes in these animals.

HIF-1α was identified in kidney cells as the transcriptional inducer of the erythropoietin gene (23, 24, 30). Subsequently, HIF-1α has been found to be ubiquitously expressed and to regulate hundreds of genes involved in metabolism, angiogenesis, and stress survival. One can view the changes directed by HIF-1α as compensatory adaptations to an oxygen-deficient environment. For instance, HIF-1α induces the expression of multiple glycolytic enzymes, including glucose transporter 1, aldolase A, enolase 1, lactate dehydrogenase, phosphofructokinase, and phosphoglycerate kinase (26). This upregulation of glycolysis decreases the cell's reliance on oxidative phosphorylation for ATP. Most studies on HIF-1α have been performed in cancer models, either cell lines or tumor tissue. Here HIF-1α has been found to be a potent promoter of angiogenesis via its upregulation of vascular endothelial growth factor and other angiogenic factors (25). More recently HIF-1α has been ascribed the role as the primary driver of the Warburg effect, or the high rates of aerobic glycolysis displayed by cancer cells (15). The close linkage between tumor

growth and metastatic potential and the ability to attract vascularization explains the intense interest in HIF signaling as it relates to cancer.

In heart, much less is known about the role of HIF-1α in pathophysiology. Recently, prolonged overexpression of HIF-1α was shown to induce a cardiomyopathy that was fully reversible upon cessation of HIF-1α expression. In the aforementioned study, reduced sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase expression was linked to the contractile dysfunction that was observed (1). In another study, cardiomyocytes treated with prolyl hydroxylase inhibitors to induce HIF-1α reduce ATP turnover by 85%, with the majority of these energy savings derived from attenuation of calcium handling/contractile activities (29). These findings indicate that HIF-1α may be an important driver of the dysfunction observed in ischemic heart disease. On the other hand, evidence has begun to emerge that HIF-1α plays a central role in cardioprotection. Ischemic preconditioning is a phenomenon where exposure of hearts to short sublethal durations of ischemia-reperfusion provides protection from further damage upon exposure to subsequent longer and normally lethal episodes of ischemic stress. In a mouse model with a null allele of the HIF-1α locus resulting in less expression of HIF-1α, a complete loss of preconditioning protection in heart was noted (3). In another recent study, small-interfering RNA (siRNA) repression of HIF-1α was found to attenuate preconditioning protection (6). Several reports have also shown that the use of pharmacological prolyl hydroxylase inhibitors to induce HIF-1α levels confers cardioprotection in several disparate cardiac model systems (19, 22, 28, 31). Similarly, administration of siRNA, which targets PHD2 (16) or genetic models where PHD2 is ablated, proves cardioprotective (17). Because PHDs have targets in addition to HIF-

1α, such as Iκkinase-1 (5), studies where PHD is inhibited do not necessarily prove HIF-1α involvement. Nonetheless, taken together, these recent findings strongly indicate a prominent role for HIF-1α in preconditioning and cardioprotection. Less clear are the HIF-1α-induced mechanisms that are important in providing protection from ischemia-reperfusion injury in heart. In these studies, we compare the ischemic stress tolerance of hearts that are induced to express HIF-1α-PPN in varying amounts and durations to begin to probe the aforementioned questions.

## EXPERIMENTAL PROCEDURES

*Reagents:* Sterile oxyrase was purchased from Oxyrase (Mansfield, OH). Tetramethylrhodamine (TMRM) was purchased from Life Technologies (Grand Island, NY). 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Rabbit polyclonal primary antibody against HIF-1α was obtained from Novus Biologicals (Littleton, CO), whereas rabbit monoclonal primary antibody against glycogen synthase was obtained from Cell Signaling (Danvers, MA). Goat anti-rabbit secondary antibodies were purchased from Millipore (Billerica, MA) and Cell Signaling. Doxycycline hydrochloride was purchased from RPI (Mount Prospect, IL).

*Animal model*: B6C3F1 mice containing the HIF-1α-PPN transgene have been previously described by Bekeredjian et al. (1). All mice used were males between 2 and 3.5 months of age and were routinely maintained on a 625 mg/kg doxycycline-replete diet (Harlan Research Laboratories, Madison, WI). In experiments requiring 2 days of HIF-1α expression (2D), mice were switched from doxycycline food to doxycycline-replete water containing 73 mM sucrose (Mallinckrodt Baker, Phillsburg, NJ) and 0.416

mM doxycycline hydrochloride for 2 days followed by maintenance of mice on regular food and water for two additional days. In experiments requiring 6 days of HIF-1α expression (6D), mice were maintained on doxycycline-free food and water for 5–7 days before experimentation. Wildtype (WT) B6C3F1 mice were obtained from Harlan. Animals were handled in accordance to a protocol reviewed and approved by the East Tennessee State University Committee on Animal Care.

*Isolation of cardiomyocytes from adult mice:* Myocytes from adult mice hearts were isolated according to procedures described by O'Connell et al. (18). Isolated myocytes were suspended in 10 ml of plating medium with 25 μM blebbistatin and incubated on plates that were precoated with matrigel (BD Biosciences, Rockville, MD) diluted 1:40 in DMEM-F-12 (GIBCO, Grand Island, NY) for 1 hour in 5% $CO_2$. After incubation in plating medium, myocytes were maintained on culture medium with 25 μM blebbistatin. Experiments using adult mice cardiomyocytes were performed on the same day as cell isolation.

*Preparation of mice heart homogenates*: Hearts from wildtype (WT), non-induced (Non-I), 2D, and 6D mice were excised and washed briefly in PBS to remove excess blood. Hearts were then immediately clamped with a set of tongs that were pre-chilled in liquid nitrogen. They were then ground into a fine powder using a mortar and pestle under liquid nitrogen. The powdered heart tissue was homogenized in RIPA buffer composed of 50 mM Tris·HCl, pH 7.4 (Calbiochem, Darmstadt, Germany), 1% v/v Triton X-100 (Fisher, Pittsburgh, PA), 1% w/v sodium deoxycholate (Fisher), 0.1% w/v SDS (EMD, Billerica, MA), and 1 mM EDTA (Fisher) with 1:40 protease inhibitor cocktail

mix (Sigma, St. Louis, MO). The homogenates were centrifuged at 12,000 *g* at 4°C for 10 minutes. The supernatant was collected. Protein concentration for the supernatant was determined using the Pierce BCA protein assay kit from Thermoscientific (Rockford, IL) according to the manufacturer's protocol.

*SDS-PAGE and western blot*: HIF-1α and glycogen synthase expression were evaluated using standard SDS-PAGE and Western blotting techniques. Protein samples were separated using SDS-PAGE in Pierce Tris-HEPES-SDS 4–20% precast polyacrylamide gels (Thermoscientific). Proteins were transferred to polyvinylidene difluoride membranes (BioRad, Richmond, CA) at 75 volts for 2 hours. After transfer, Ponceau S (Sigma) staining was used to ensure complete transfer and equal protein loading. Membranes were blocked in 5% nonfat dry milk in TBS with 0.1% Tween 20 (TBS-T) for 1 hour at room temperature. HIF-1α expression was probed using a rabbit polyclonal primary antibody diluted 1:500 in TBS-T, and glycogen synthase expression was probed with a rabbit monoclonal primary antibody at 1:1,000 dilution in TBS-T. Both membranes were incubated at 4°C overnight and washed for 5 minutes in TBS-T (5x) before incubation with goat anti-rabbit horseradish peroxidase-conjugated (HRP) secondary antibody. Protein bands were detected using the Pierce supersignal chemiluminescence substrate (Thermoscientific) in the G:Box fluorescence and chemiluminescence imaging system (Syngene, Frederick, MD). Densitometry was performed using ImageJ (National Institutes of Health, Bethesda, MD).

*Langendorff perfusion:* Hearts were retrograde perfused through the aorta with Krebs buffer containing (in mM): 118.5 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 24.8

$NaHCO_3$, 2.5 $CaCl_2$, and 10.6 glucose. The buffer was equilibrated with 95% $O_2$ and 5% $CO_2$ and maintained at 37°C. A fluid-filled silicon balloon was inserted in the left ventricle through the mitral valve for left ventricular developed pressure (LVDP) measurement with a pressure transducer (AD Instruments, Dunedin, New Zealand). Balloons were fabricated using methods previously published (14). Hearts were allowed to stabilize during a 25 minute baseline period after which, function, tissue viability, lactate accumulation, ATP, and ADP levels were evaluated after hearts had been subjected to various ischemia-reperfusion protocols. For measurement of lactate production during ischemia, the perfusate during the first 5 minutes of reperfusion was collected. Lactate content was measured colorimetrically (450 nm) in a 96-well plate format following instructions from the Biovision lactate assay kit (Biovision, Milpitas, CA).

*Evaluation of tissue viability after ischemia-reperfusion:* After the initial stabilization period, hearts were subjected to 30 minutes of ischemia and 60 minutes of reperfusion. At the end of the protocol, the hearts were perfused with 1% TTC and then taken off the cannula to incubate for 15 minutes at 37°C. Afterwards, the hearts were sliced transversely, and images of the transverse slices were taken using a Microtek film scanner (Microtek International, Hsinchu, Taiwan). The viable (stained dark orange) and non-viable (unstained yellow) sections were analyzed using Adobe Photoshop (Adobe Systems, San Jose, CA).

*Measurement of pre-ischemic glycogen reserves:* For glycogen content, frozen powdered heart tissue was transferred to an Eppendorf tube and 500 µl of $ddH_2O$ was

added. The samples were immediately boiled for 5 minutes and centrifuged at 13,000 RPM. Protein concentration was determined using the Pierce BCA assay kit (Thermoscientific). Glycogen content was measured colorimetrically at 570 nm on a 96-well plate format following instructions provided with the Biovision glycogen assay kit (Biovision).

*Measurement of nucleotides:* ATP and ADP measurements in hearts subjected to ischemia were done using high-performance liquid chromatography (HPLC) following a method established by Giannattasio et al. (7). At the end of the respective perfusion protocol (pre-ischemic, 5, 10, 20, or 30 minutes of ischemia; or 30 minutes of ischemia followed by 30 minutes of reperfusion), hearts were freeze-clamped and then pulverized to a fine powder using a mortar and pestle. Next, the frozen heart tissue was homogenized in 400 µl of 4% perchloric acid (Alfa Aesar, Ward Hill, MA) to extract nucleotides. The homogenate was incubated on ice for 20 minutes. Following incubation, the homogenate was centrifuged at 15,000 *g* for 15 minutes at 4°C. The supernatant containing total nucleotides was collected and stored in −80°C until further processing. The remaining tissue pellet was lyophilized to obtain the dry tissue weight.

Before measurement of ATP and ADP on HPLC, the samples containing total heart nucleotides were neutralized in a solution consisting of 4/5 volume 2 M KOH (Fisher) and 1/5 volume 1 M $KH_2PO_4$ (MP Biomedical, Solon, OH). After neutralization, the samples were incubated on ice for 10 minutes and centrifuged for 15 minutes at 15,000 *g* at 4°C. The resulting supernatant was collected and filtered through a 0.22-µm syringe filter (Millipore).

HPLC runs were performed using a binary gradient with increasing organic strength. The binary gradient was programmed into the LCsolutions data acquisition software (Shimadzu Scientific Instruments, Columbia, MD). The mobile phases were *buffer A*, which consists of 8 mM tetrabutylammonium hydrogen sulfate (Acros Organic, Morris Plains, NJ) and 0.1 M $KH_2PO_4$ (MP Biomedical). *Buffer B* consists of 8 mM tetrabutylammonium hydrogen sulfate (Acros Organic) and 0.1 M $KH_2PO_4$ (MP Biomedical) with the addition of 30% $CH_3CN$ (Fisher). Both mobile phase buffers were pH to 6. Sample (100 µl) was injected into a 20-µl loop, and peak detection was done using the SPD-M20A diode array detector at 254 nm (Shimadzu Scientific Instruments) and recorded with the LCsolutions data acquisition software. The areas of the peaks corresponding to ATP and ADP were integrated using the LCsolutions postrun analysis program (Shimadzu Scientific Instruments). Total ATP and ADP content expressed in units of µmole was calculated from the respective calibration curves and normalized against the amount of dry tissue collected (grams).

*Measurement of mitochondrial membrane potential during anoxia and metabolic inhibition:* Adult mice cardiomyocytes were plated on 50-mm MatTek dishes (MatTek, Ashland, MA) and incubated in 1.5 ml of culture medium as described by O'Connell et al. (18) with 200 nM TMRM at 37°C and 5% $CO_2$. After 30 minutes of incubation in 200 nM TMRM, the culture medium was substituted with 50 nM TMRM in PBS plus 5 mM glucose and 10 mM succinate. Before imaging, a cover slip was placed over the cells, and 15 µl of sterile oxyrase were added. Oxyrase selectively removes oxygen, creating depletion of oxygen in the cardiomyocyte's immediate surroundings. Fluorescent imaging was performed on the Zeiss Axio Observer Z1 inverted fluorescent microscope

(Zeiss, Gottingen, Germany). Images for cardiomyocytes from all mice groups were captured using the Zeiss AxioCam MRm monochrome digital camera (Zeiss) at $t_0$, the time point immediately after culture medium was substituted with PBS plus 5 mM glucose, 10 mM succinate, and 50 nM TMRM. Subsequent images were captured after 0.5, 1, 1.5, and 2 hours of incubation with oxyrase. Images were analyzed with the Zeiss AxioVision 4.8.2 software. The number of polarized myocytes (i.e., those exhibiting TMRM fluorescence) was counted at each time point of oxyrase incubation and was expressed as a percent of polarized myocytes prior to anoxia. For measurement of mitochondrial membrane potential during inhibition of oxidative phosphorylation and glycolysis, isolated myocytes were incubated with 200 nM TMRM in culture medium for 30 minutes. After this period, culture medium was replaced with PBS plus 50 nM TMRM, 2 mM sodium cyanide (Mallinckrodt Baker), and 5 mM 2-deoxyglucose (2-DG; Acros), and a series of fluorescent images were taken at 0, 15, 30, 45, and 60 minutes of incubation. The number of polarized rod-shaped cells at each incubation time point (i.e., those displaying TMRM fluorescence) was counted and expressed as a fraction of polarized cells prior to cyanide and 2-deoxyglucose treatment.

*Statistical analysis:* Data reported here are expressed as means±SEM. Statistical analysis was performed using one-way ANOVA followed by Student-Newman-Keuls post hoc testing using GraphPad Prism 5 (La Jolla, CA).

## RESULTS

### *HIF-1α-expressing hearts tolerate ischemia better.*

Hearts derived from WT and transgenic animals induced to express HIF-1α for the indicated times were retrograde perfused and subjected to 30 minutes of ischemia followed by reperfusion to allow recovery. No differences in the spontaneous beating rate or LVDP were noted between HIF-1α-expressing and control hearts before ischemia. Upon cessation of perfusion, the ischemic contracture was found to be more slowly developing and of a lower ultimate magnitude in 2D- and 6D-induced HIF-1α hearts (Figure 2.1). Recovery of function following ischemia was remarkably higher in Non-I, 2D, and 6D HIF-1α-induced hearts compared with WT hearts (Figure 2.2*A*). The magnitude of ischemic contracture was significantly lower for 2D-induced and 6D-induced hearts, but not Non-I hearts, compared with the WT hearts (Figure 2.2*B*).

Next, tissue viability after the ischemic challenge was assessed using TTC staining. Tissue viability was found to correlate well with the enhanced functional recovery of the hearts (Figure 2.3, *A* and *B*). Somewhat surprisingly, the recovery of pre-ischemic LVDP and tissue viability of HIF-1α-PPN Non-I hearts was equivalent to the 2D-induced and 6D-induced hearts (Figures 2.2 and 2.3). This finding caused us to examine the possibility of HIF-1α-PPN "leakage" in the hearts of animals maintained on a doxycycline-replete diet. Indeed, we find that, while Non-I heart extracts contain far less HIF-1α protein than their induced counterparts, they do have higher levels than WT hearts (Figure 2.3*C*). Perhaps this should not have been unexpected given that HIF-1α-PPN has been modified to be stable under normoxic conditions via the substitution of critical amino acids within its degradation domain. Thus, any small expression leakage will result in some accumulation of the stable HIF variant. The results indicate that, while HIF-1α-PPN expression is much lower in the Non-I hearts, it is sufficient to protect

to a similar extent, at least against this stress protocol. In contrast, the moderation of ischemic contracture appeared to require the higher, induced levels of HIF-1α (Figure 2.2*B*).

***HIF-1α causes glycogen synthase induction, glycogen accumulation, and increased glycolytic flux during ischemia.***

Because ischemic contracture has been reported to be delayed and reduced in magnitude by higher glycogen reserves, we next examined the effects of HIF-1α expression on glycogen levels in the HIF-expressing and WT hearts. Although a trend was observed for higher glycogen reserves in all the hearts expressing HIF-1α-PPN, only the 6D HIF-induced mouse hearts had significantly higher levels of glycogen stores compared with WT and Non-I hearts (Figure 2.4*A*). Given the higher glycogen stores at the initiation of ischemia, we sought to estimate the glycolytic flux during the 30 minutes of no-flow ischemia. Accordingly, the next experiment measured the lactic acid accumulated during the 30 minutes of ischemia that is released during the first 5 minutes of reperfusion (Figure 2.4*B*). As with glycogen stores, lactic acid release was significantly elevated in the 6D HIF-induced hearts compared with hearts from WT and the Non-I HIF-1α-PPN mice. Recent reports indicating that glycogen synthase is a HIF-1α-responsive gene might explain the higher levels of glycogen stores in the HIF-expressing hearts. Consistent with this, we find significantly elevated glycogen synthase protein levels in HIF-1α-PPN transgenic mice hearts (Figure 2.4*C*). Interestingly, glycogen synthase protein levels are elevated to a similar extent in Non-I hearts as those induced for 2 and 6 days. The discrepancy between glycogen synthase and glycogen levels among the experimental groups probably reflects the complex array

of factors that ultimately determine tissue glycogen levels. Nonetheless, these results showing that HIF-1α directs induction of glycogen synthase and glycogen accumulation in adult heart are important findings. Given that glycogen granule accumulation is a hallmark of the hibernating myocardium (9), it indicates that HIF-1α may be a driver of this pathophysiological phenotype.

***The upregulation of glycolytic metabolism does not explain the protection afforded by HIF-1α.***

The well-known ability of HIF-1α to induce many of the enzymes that comprise the glycolytic pathway when coupled to elevated glycogen stores suggests an obvious avenue to achieve the cardioprotection observed in the HIF-1α-PPN hearts. Mobilization of the glucose stores, and enhanced glycolysis and its attendant ATP production during stoppage of perfusion, is consistent with the moderation of ischemic contracture and increased lactic acid release that we observe in HIF-expressing hearts. To address the issue of glycolytically produced ATP moderating the contracture and the damage sustained during ischemia, hearts were flash-frozen at intervals during ischemia and extracted, and nucleotide contents were measured with HPLC. Examples of HPLC chromatograms showing nucleotides and their metabolites are provided in Figure 2.5. Hearts derived from 2D- and 6D-induced HIF-1α-PPN hearts maintained significantly elevated levels of ATP at 20 and 30 minutes of ischemia compared with WT control hearts (Figure 2.6*A*). Among the experimental groups, only 6D-induced HIF-1α-PPN hearts maintained significantly elevated ATP:ADP ratio at any time point of ischemia (Figure 2.6*B*). The comparison of ATP depletion between Non-I HIF-1α-PPN and WT hearts showing no differences in the degree of ATP depletion is most

interesting given the high degree of ischemic tolerance the Non-I hearts display. These findings call into question the relative importance of the compensatory increases in glucose stores and glycolytic enzymes in the protective phenotype of the HIF-1α-PPN hearts. Other mechanisms besides increasing glycolytic flux during ischemia are suggested to be employed by HIF-1α to protect the myocardium. The next series of experiments were designed to begin to probe alternative mechanisms of protection.

Previously, we found that the ability of mitochondria to maintain polarization during simulated ischemia is strongly associated with protection in the heart cell (29). Thus, we tested whether isolated adult cardiomyocytes from HIF-1α-PPN hearts also display improved mitochondrial function under similar conditions. Adult cardiomyocytes were isolated from WT, Non-I, 2D- and 6D-induced HIF-1α-PPN hearts and subjected to anoxia. Mitochondrial polarization was monitored with TMRM potentiometric dye for 2 hours under these conditions. We find that HIF-1α-PPN myocytes maintained mitochondrial polarization in significantly greater numbers than WT cells, with 6D-induced cardiomyocytes performing best, being little affected over the 2 hour period (Figure 2.7). In the next experiment, the dependency of protection on enhanced glycolytic flux was directly addressed. Myocytes derived from WT, Non-I, 2D- and 6D-induced HIF-1α-PPN hearts were treated with cyanide and 2-DG to block both oxidative phosphorylation and glycolysis, and mitochondrial polarization was followed for 1 hour. We find that HIF-1α-expressing myocytes are able to maintain mitochondrial membrane potential under these conditions to a significantly greater extent than WT myocytes (Figure 2.8). This finding causes us to conclude that the well-known compensatory increase in glycolytic capacity that is induced by HIF-1α is not necessary for the

ischemic tolerance conferred by HIF-1α and points to the existence of additional mechanisms that center on the mitochondrion.

## DISCUSSION

Twenty years after its discovery, HIF-1α is well established to play a central role in the cellular response to hypoxia; however, much less is known about its cardiospecific effects. In these studies, we show that forced expression of a stabilized mutant version of HIF-1α confers robust protection to adult heart in a common *ex vivo* model of ischemia-reperfusion injury. Given the prominence of ischemic stress in the constellation of pathologies grouped under the umbrella of heart disease, it is of great interest to understand the mechanisms the cardiomyocyte employs to protect itself against hypoxia stress. Certainly HIF's actions in protecting the myocyte from irreversible damage during ischemia can be regarded as beneficial. On the other hand, evidence also exists that ascribes a pathological role to HIF-1α in the context of decreased myocardial function during chronic hypoxia such as occurs in ischemic heart disease. Indeed HIF's pathological and protective mechanisms are likely to overlap, in that decreased function and lower ATP turnover represent powerful protective maneuvers when oxygen becomes limited (11, 12). In this vein, we should note that HIF-1α-PPN induction for up to 6 days did not change the basal contractile characteristics (i.e., LVDP when paced, or spontaneous heart rate, data not shown) of mice hearts when they were examined *ex vivo* with Langendorff perfusion. Thus, the robust ischemic protection afforded by HIF-1α expression was not accompanied by any indication of pathological response in the time frames examined in this study.

The most prominent effect of HIF-1α-PPN expression on the response of the perfused heart to 30 minutes of ischemia is an attenuated ischemic contracture. Sometimes referred to as “stone heart,” the underlying mechanisms governing contracture are not completely understood (8). Initiation of contracture is closely tied to the rate of ATP depletion and exhaustion of internal glycogen stores (8). It is important to note, however, that the severity of ischemic contracture does not correlate with myocardial protection or recovery of function in heart. This is best illustrated by the fact that ischemic preconditioning, which is highly protective, shortens the time until onset of contracture probably through depletion of glycogen stores. This has led to the suggestion that glycogen and high rates of glycolysis and acidification during ischemia are detrimental to cellular recovery. The degree of severity and length of the ischemic insult probably determine whether mobilization of glycogen stores and glycolytic ATP production are effectively beneficial or detrimental to tissue viability (4). For instance, with chronic low-flow ischemia, enhanced glycolytic ATP generation greatly improves functional outcomes.

Recently, muscle glycogen synthase was shown to be a HIF-1α-inducible gene in $C_2C_{12}$ skeletal muscle myotubes and several cell lines (21). Our results show that HIF-1α elevates glycogen synthase in adult heart cells and leads to glycogen accumulation, especially in 6D HIF-1α-induced hearts, where significant elevations of glycogen synthase, glycogen, and lactic acid production during ischemia were noted. Of importance, the enhanced glycolysis was reflected in a significant diminishment of ATP depletion in the 6D-induced hearts during ischemia. The finding that HIF-1α is sufficient to cause glycogen accumulation in the adult myocardium is also of significance.

Glycogen accumulation is a hallmark of hibernating myocardium. Given previous findings that HIF-1α suppresses oxidative phosphorylation (20), ATP turnover, and contractile function (29), a strong circumstantial argument can be made that HIF-1α may play a large role in directing the hibernating phenotype in heart. As mentioned above, however, we were unable to detect any changes in contractile function or oxidative respiration in 6D HIF-induced hearts. Specifically, LVDP, dP/d*t*, and $O_2$ consumption were measured in WT, Non-I, and 2D- and 6D-induced hearts, whereas pacing was increased in 200-beat/minute step increments from 300 to 1,300 beats/minute. No significant changes in these parameters were found between the hearts in any of the experimental groups (data not shown). We are presently uncertain why HIF-1α-expressing hearts fail to show any evidence of reduced oxidative phosphorylation (29) or contractile function (1) noted in other model systems. Several possible explanations are currently being tested but are beyond the scope of these studies, which have focused upon the protective effects of HIF-1α-directed changes.

In these studies, we have exploited our inducible HIF-1α-PPN transgenic system to provide something akin to the dose-response curve, where the influence of HIF-1α is absent in the wildtype: < in Non-I << 2D-induced < 6D-induced derived hearts. This pseudo dose-response yielded interesting insights into the biology of the compensatory responses elicited by HIF-1α. We examined pre-ischemic glycogen reserve, glycolytic activity, and ATP depletion kinetics during ischemia in WT, Non-I, and 2D- and 6D-induced mice hearts. We found that the Non-I HIF-1α-PPN hearts did not show significant increases in pre-ischemic glycogen store, glycolytic flux during 30 minutes of ischemia, or increased ability to maintain ATP levels during various times of ischemia

compared with WT hearts. Nonetheless, Non-I hearts were protected to the same extent as hearts induced to express HIF-1α for 2 and 6 days. This suggests that, while increasing glycogen reserves before, and glycolytic flux during, ischemia clearly represent a compensatory response; it may not comprise the primary mechanism of protection to the total global ischemia employed in these studies.

Similar to these studies, our work examining the cardioprotective mechanisms of the $O_2$ sensor in neonatal cardiomyocytes called into question the central importance of the induction of glycolytic capacity and glycogen accumulation in protection (28). In previous work, we found that HIF-1α expression, albeit as the result of dimethyloxalylglycine treatment rather than genetic manipulation, led a persistent ability to maintain mitochondrial polarization during anoxia or cyanide poisoning, even when glycolysis was blocked or the reverse mode of ATP synthase was inhibited. After extensive analysis, we ultimately concluded that fumarate was used as an alternate terminal electron acceptor to allow electron flux to continue through complex I during anoxia or cyanide poisoning (29). In the studies shown in Figure 2.7 and Figure 2.8 isolated adult heart cells were tested in vitro to determine whether the protection observed in HIF-expressing hearts would translate into stabilized mitochondrial polarization during anoxia. In both anoxic conditions permissive for glycolysis, and in conditions where both oxidative phosphorylation and glycolysis are blocked (cyanide and 2-DG present), mitochondrial membrane potential was significantly better maintained in the HIF-1α-expressing cardiomyocytes. Taken together, these results reveal that HIF-1α can provide ischemic cardioprotection via mechanisms independent of compensatory increases in glycolytic flux and ATP preservation. Studies are

underway to quantitatively measure the flux through fumarate respiratory pathways that HIF-1α induces.

Clearly HIF-1α is initiating a complex multifactorial compensatory program that equips the adult cardiomyocyte with remarkable tolerance to hypoxic stress. The most well-known HIF-1α-driven response to hypoxia is the induction of glycolytic pathway enzymes (25). Our data confirm that glycolytic capacity is increased by HIF-1α in adult murine cardiomyocytes and that this reserve is tapped during acute ischemia. Somewhat surprising, the findings also call into question the central importance of this increased glycolytic capacity as the central mechanism leading to the powerful protection conferred by HIF-1α in the acute no-flow ischemic protocol that we employ. Rather, isolated cell studies implicate mechanisms that preserve mitochondrial function during ischemia in the powerful cardioprotection that is observed.

**FIGURES**

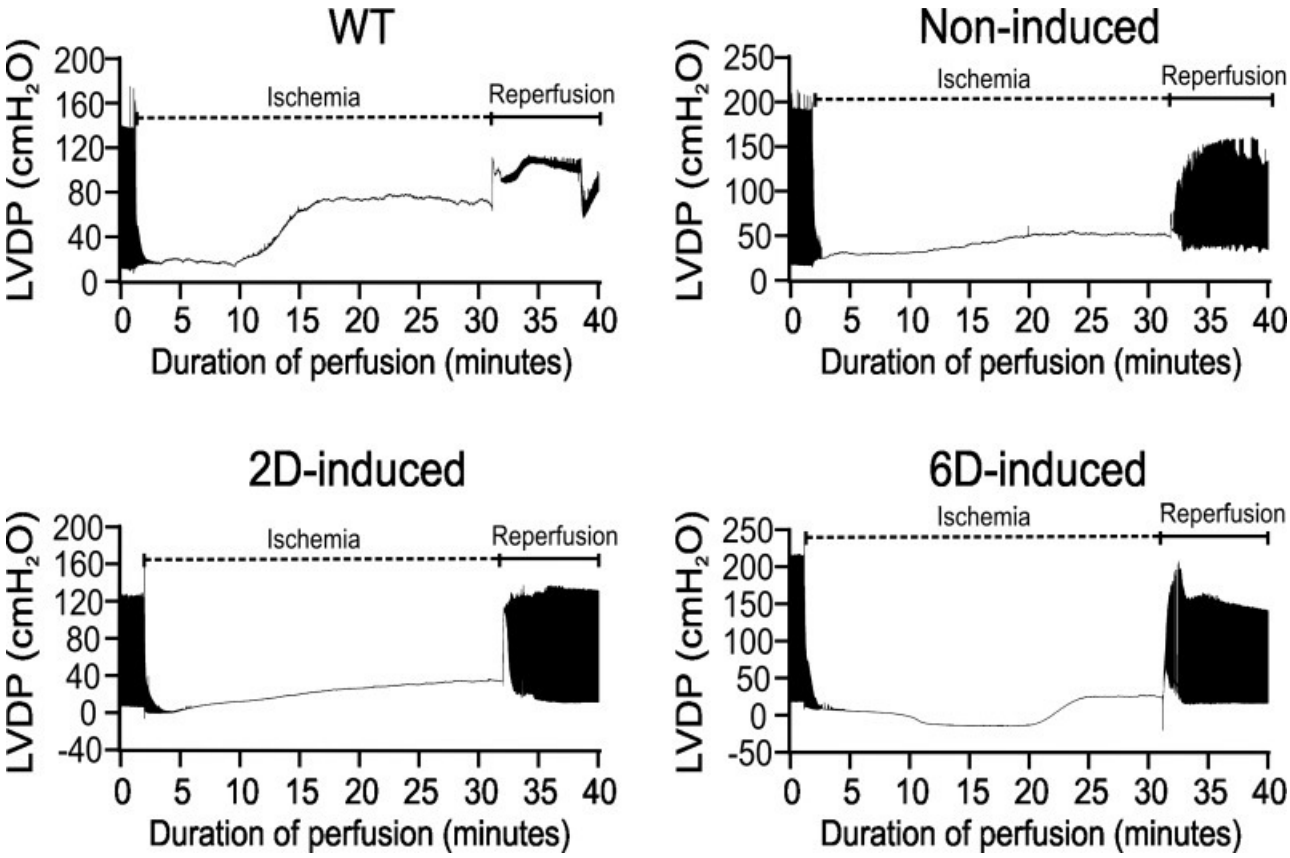



Figure 2.1: *Response of murine hearts with varying degrees of hypoxia-inducible factor-1α (HIF-1α) expression to 30 minutes of ischemia, followed by reperfusion*. Shown are exemplary condensed pressure tracings from Langendorff-perfused hearts derived from wildtype (WT) and HIF-1α-PPN mice that were not induced (Non-I) and induced to express HIF-1α for 2 days (2D induced) or 6 days (6D induced). Hearts were subjected to 30 minutes of ischemia following pre-equilibration for at least 20 minutes, whereupon they were allowed to recover. In some hearts, the left ventricular balloon was deflated during ischemia and reperfusion. In the example shown here, the balloon was allowed to remain inflated so that ischemic contraction could be measured. LVDP, left ventricular developed pressure.

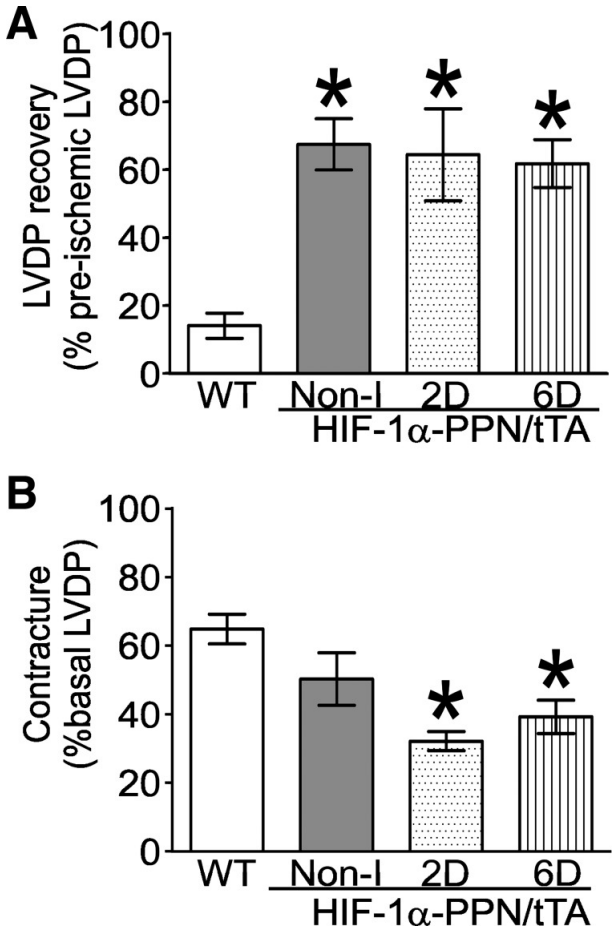



Figure 2.2: *Quantification of cardiac functional recovery after ischemia of the experiment shown in Figure 2.1. A*: recovery of LVDP after 30 minutes of ischemia. *B*: ischemic contracture expressed as a percent of basal LVDP. **P* < 0.05 vs. WT; *n* = 6–8 hearts.

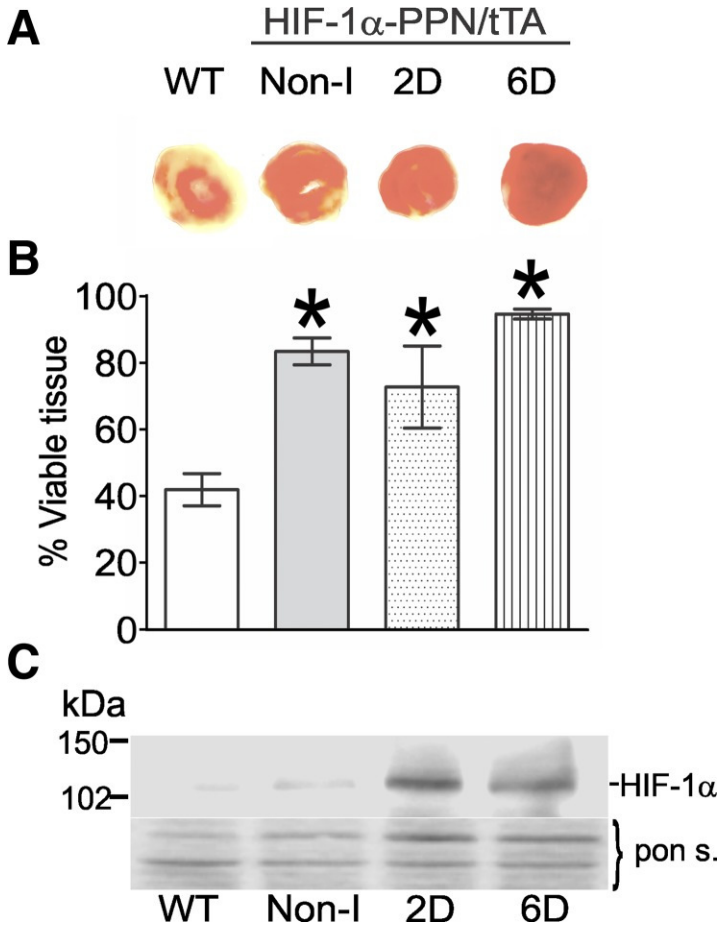



Figure 2.3: *HIF-1α expression limits myocardial ischemic damage. A*: typical triphenyltetrazolium chloride (TTC) staining pattern in WT, non-induced, 2D-induced, and 6D-induced mouse hearts post-ischemic reperfusion insult; note that viable tissue stains a darker orange color. *B*: viable tissue was determined by TTC staining in perfused hearts following 30 minutes of ischemia and 60 minutes of reperfusion. The percent viable tissue was quantified using images of transverse sections of heart ventricles and Adobe Photoshop software. *$P < 0.05$ vs. WT. *C*: representative Western blot showing HIF-1α protein levels in protein extracts from hearts in the indicated treatment groups.

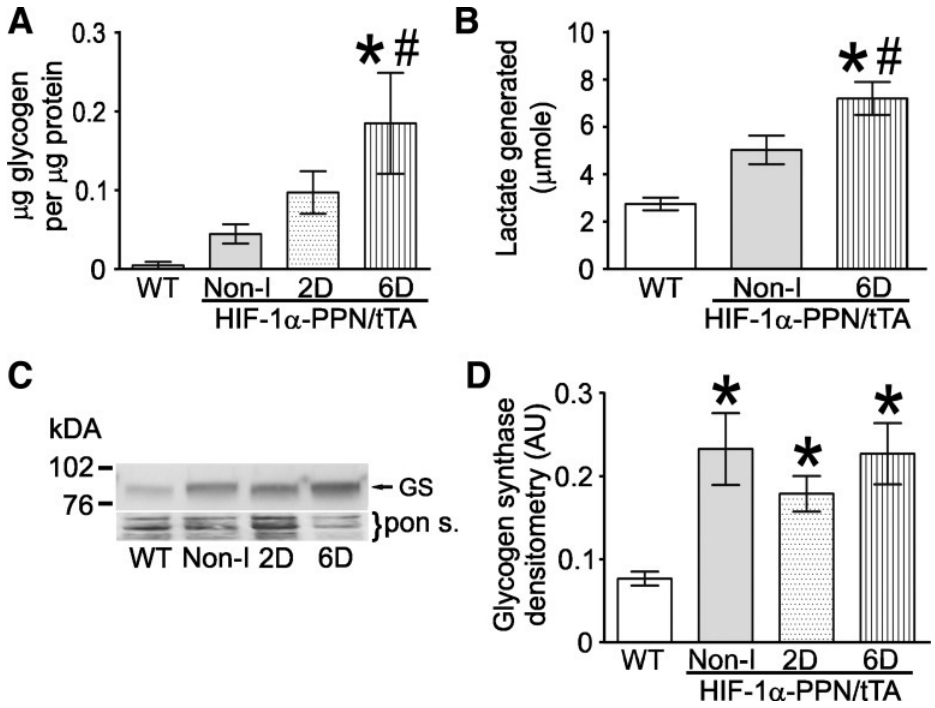
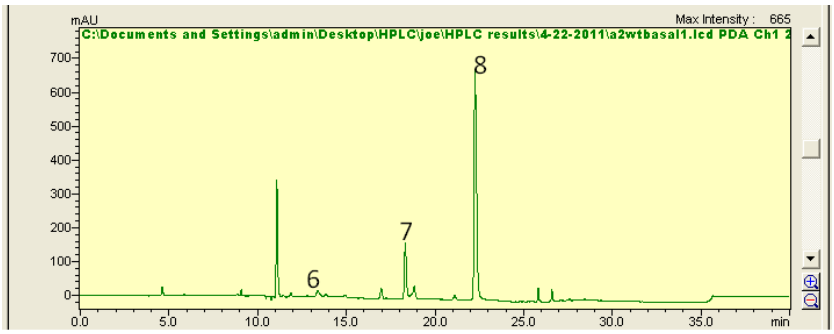



Figure 2.4: *Glycogen reserves are increased in HIF-1α-expressing hearts. A*: measurement of glycogen content in WT, non-induced, 2D-induced, and 6D-induced mouse hearts ($n$ = 4 hearts). *B*: accumulated lactate was measured in the perfusion buffer effluent collected during the initial 5 minutes of reperfusion after 30 minutes of ischemia. *C*: Western blot showing glycogen synthase protein levels in WT, non-induced, 2D-induced, and 6D-induced mouse heart extracts. *D*: densitometry analysis of the glycogen synthase protein band intensity from the Western blot experiment shown in *C* ($n$ = 4). *$P$ < 0.05 vs. WT. #$P$ < 0.05 vs. non-induced.

**A**

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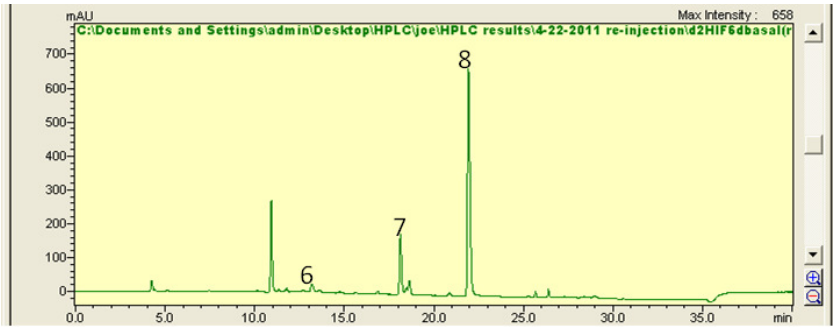


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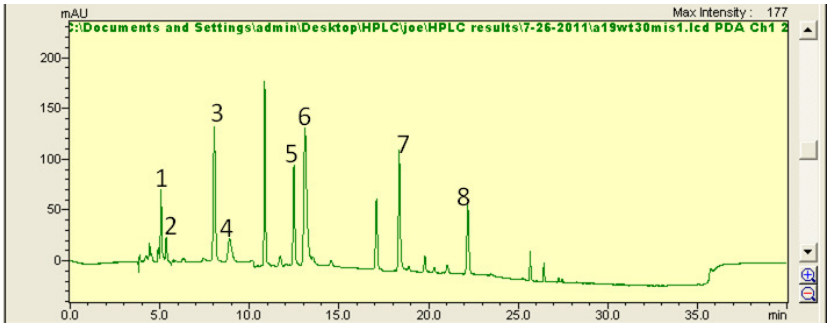


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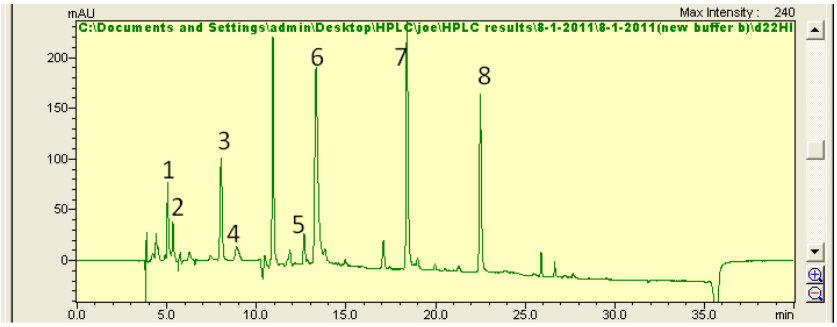


Figure 2.5: *Examples of HPLC chromatograms showing the content of adenine nucleotides, nucleosides, and nucleobases in mouse hearts. A*: pre-ischemic wildtype mouse heart. *B*:

pre-ischemic 6 day HIF-1α induced (6D) mouse heart. *C*: wildtype mouse heart after 30 minutes of ischemia. *D*: 6 day HIF-1α induced (6D) mouse heart after 30 minutes of ischemia. Peaks: (1) hypoxanthine {RT=5.05 minutes}, (2) xanthine {RT=5.34 minutes}, (3) inosine {RT=8.02 minutes}, (4) IMP {RT=8.89 minutes}, (5) adenosine {RT=12.66 minutes}, (6) AMP {RT=13.32 minutes}, (7) ADP {RT=18.40 minutes}, (8) ATP {RT=22.49 minutes}. RT stands for retention time for the indicated peak.

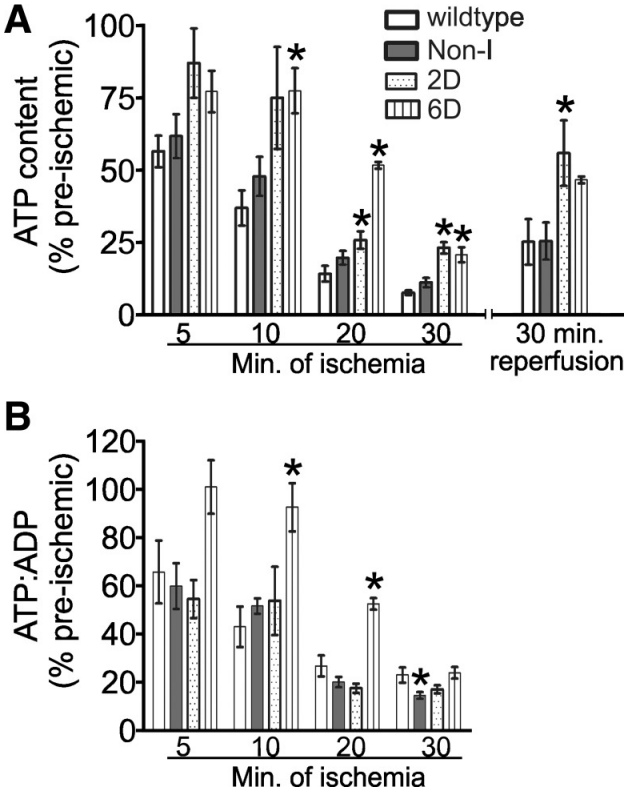


Figure 2.6: *ATP levels are better maintained during ischemia in hearts where HIF-1α expression has been induced*. Hearts were freeze-clamped and extracted at the indicated time points of ischemia. ATP and ADP levels were measured by reverse-phase HPLC (see experimental procedures). *A*: ATP content in hearts subjected to increasing durations of ischemia. ATP levels are expressed as a percentage of pre-ischemic values within all experimental groups. Pre-ischemic ATP values were 26.4 ± 3.7, 27.7 ± 8.7, 19.4 ± 11.2, and 21.4 ± 2.4 μmole/g dry tissue in WT, non-induced, 2D-induced, and 6D-induced mouse hearts, respectively. *B*: the ATP-to-ADP ratio expressed as a percentage of the pre-ischemic values, which are 3.3 ± 0.6, 4.5 ± 1.0, 4.8 ± 0.3, and 3.1 ± 0.6 for WT, non-induced, 2D-induced, and 6D-induced mouse hearts, respectively. The data represent means±SEM ($n$ = 3–9). *$P$ < 0.05 vs. WT.

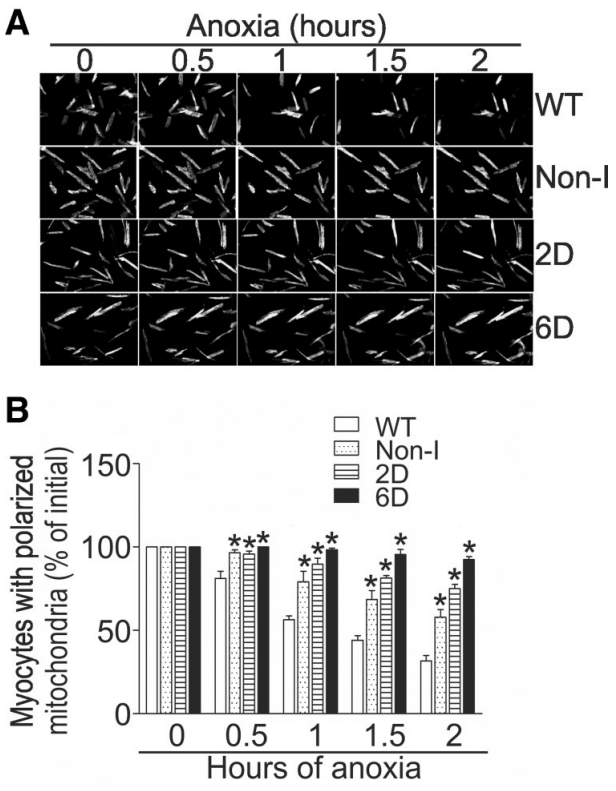


Figure 2.7: *Mitochondrial membrane polarization is better maintained in HIF-1α-expressing adult cardiomyocytes during anoxia*. *A*: typical TMRM fluorescent image of WT, non-induced (Non-I), 2 day-induced (2D), and 6 day-induced (6D) mouse heart cardiomyocytes after 0, 0.5, 1, 1.5, and 2 hours of anoxia. *B*: cells with polarized mitochondria relative to the amount before anoxia expressed as means±SEM ($n$ = 3–8). *$P$ < 0.05 vs. WT.

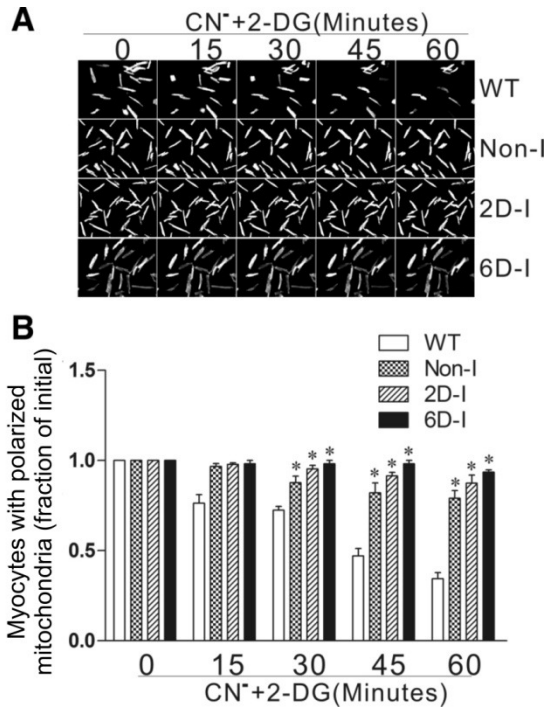


Figure 2.8: *HIF-1α-expressing cardiomyocytes maintain polarized mitochondria when both oxidative phosphorylation and glycolysis is blocked*. *A*: representative TMRM fluorescent images of cardiomyocytes obtained from WT, Non-I, 2D-induced, and 6D-induced mice after 0, 15, 30, 45, and 60 minutes of cyanide ($CN^-$) and 2-deoxyglucose (2-DG) treatment to block oxidative phosphorylation and glycolysis, respectively. *B*: number of rod-shaped cells with polarized mitochondria relative to the amount prior to cyanide and 2-DG treatment expressed as means±SEM ($n$ = 3). *$P$ < 0.01 vs. WT.

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# CHAPTER 3

## HIF-1α IN THE HEART: EXPRESSION OF PURINE NUCLEOTIDE CYCLE ENZYMES


## SUMMARY

Previous studies have shown that activation of HIF-1α signaling conferred the ability for cardiomyocytes to use fumarate as an alternative terminal electron acceptor to sustain anoxic mitochondrial electron transport chain (ETC) activity. Further investigations revealed that the source of fumarate was the nucleotide metabolic pathway known as the purine nucleotide cycle (PNC). In those studies, HIF-1α signaling was induced via treatment with prolyl hydroxylase (PHD) inhibitor dimethyloxaloylglycine (DMOG). However, as PHD activates pathways in addition to HIF-1α, the direct effect of HIF-1α on PNC activity cannot be established. Here, we utilized wildtype mouse hearts and hearts in which HIF-1α is overexpressed to establish whether HIF-1α directly induces the enzymes in the PNC. We report that HIF-1α induces the entry point of the PNC, AMP deaminase 2 (AMPD2) while not affecting downstream reaction steps adenylosuccinate synthetase (ADSS) and adenylosuccinate lyase (ADSL). During ischemia, the inability for glycolysis to meet ATP demands of the heart leads to an imbalance between ATP consumption and ATP synthesis. Thus, ATP in the heart is depleted and AMP accumulates. AMP can be metabolized by AMP deaminase to IMP. Subsequent cycling of IMP through the PNC regenerates AMP. Thus, the PNC acts as a conservation pathway for the heart's nucleotide pool. The induction of glycolysis in conjunction with the PNC by HIF-1α may preserve the heart's adenine nucleotide resources during ischemic insult. These considerations provide the motivation for us to

pursue a full understanding of how HIF-1α influences nucleotide metabolism in the ischemic heart and results will be presented in the next chapter.

## INTRODUCTION

Studies pioneered by Peter Hochachka demonstrated that succinate is a major metabolic end product associated with anoxia (3, 4). Mechanisms leading to succinate production via the conversion of succinyl-CoA and reduction of fumarate have been proposed by Hochachka (Figure 3.1) (3). Studies by Weinberg et al., Hohl et al., and Sridharan et al. have demonstrated that in kidney and cardiomyocytes, succinate synthesis under anaerobic conditions occurs mainly via fumarate reduction at mitochondrial complex II as indicated in Figure 3.1 (5, 10, 12).

Sridharan et al. demonstrated that activation of the HIF-1α signaling cascade in neonatal mice cardiomyocytes, albeit via treatment with prolyl hydroxylase inhibitor DMOG, led to enhanced succinate production during anoxia (10). These studies led to the unexpected finding that aspartate transamination as proposed by Hochachka (3) was not the source of fumarate used for succinate synthesis. The source of fumarate instead, was the nucleotide metabolic pathway known as the purine nucleotide cycle (PNC; see Figure 3.2). As ATP is depleted during anoxic stress, AMP accumulates. The series of reactions in the PNC starts when AMP is converted to IMP by AMP deaminase (Figure 3.2). IMP is subsequently metabolized by adenylosuccinate synthetase, an enzyme that uses aspartate as a co-factor to generate S-AMP (Figure 3.2). Hochachka observed that anoxia leads to a depletion in cellular aspartate (3). From studies conducted by Sridharan et al., it is clear that the decrease in aspartate is due to its metabolism by adenylosuccinate synthetase (10). S-AMP is finally

metabolized by adenylosuccinate lyase to produce AMP and fumarate (Figure 3.2). Those cardiomyocytes treated with DMOG were able to initiate electron transfer by oxidizing NADH at complex I (Figure 3. 2). The resulting electron is then taken up by fumarate at complex II, which acts as the terminal electron acceptor in place of oxygen. This mechanism leads to the synthesis of succinate and the continual pumping of $H^+$ from the mitochondrial matrix into the intermembrane space, thus allowing DMOG treated cardiomyocytes to maintain mitochondrial polarization during anoxia, leading to tolerance from anoxic stress (10).

Results presented in chapter 2 provide evidence that HIF-1α confers in cardiomyocytes, the ability to maintain mitochondrial polarization during ischemia. Studies conducted by Sridharan et al. demonstrated that HIF-1α may induce PNC activity (10). In those studies performed by Sridharan et al., HIF-1α signaling was activated via inhibition of prolyl hydroxylases (10). Prolyl hydroxylases target pathways in addition to HIF-1α (2). The aim in this portion of our studies is to examine the expression of PNC enzymes in wildtype and HIF-1α expressing hearts in order to determine whether HIF-1α directly induces the expression level of PNC enzymes.

**EXPERIMENTAL PROCEDURES**

*AMP deaminase (AMPD) mRNA expression:* RNA was extracted from wildtype mouse hearts (Harlan Research Laboratories, Madison, WI) and HIF-1α-PPN Non-I, 2D, and 6D mouse hearts using TRIzol reagent (Life Technologies, Carlsbad, CA). RNA concentration as well as integrity were determined using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). cDNA was synthesized using the Superscript III cDNA synthesis kit (Life Technologies). For each cDNA synthesis reaction, 2 μg of

RNA was added to 1.25 μM oligo$(dT)_{20}$, 50 ng random hexamers, 0.5 mM dNTP mix, 80 U RNaseOUT, 5 mM DTT, 1.25 mM $MgCl_2$, and 400 U reverse transcriptase in a final volume of 40 μl. The cDNA synthesis reaction was allowed to proceed for 1 hour at 50$^{o}$C and then terminated by heating at 70$^{o}$C for 15 minutes. Quantitative PCR was performed in triplicate for all samples in a 96 well plate format on the CFX96 real-time PCR detection system (BioRad Laboratories, Richmond,CA). 40 cycles were carried out. Each reaction consisted of 50 ng cDNA and1.5 μl of Quantitect AMPD2 or transferrin primer from Qiagen in 1x SsoFast Evagreen supermix (BioRad Laboratories). AMPD2 mRNA expression was normalized to that of reference gene transferrin and results were reported as a percent change in gene expression relative to wildtype.

*Protein expression of PNC enzymes:* Protein samples from WT and HIF-1α-PPN Non-I, 2D, and 6D mouse hearts were prepared according to methods detailed in chapter 2. AMPD2 was probed using a mouse monoclonal primary antibody diluted 1:1000 in TBS-T (Abcam, Cambridge, MA). ADSS was probed using a rabbit polyclonal antibody against ADSS diluted 1:1000 in TBS-T (Proteintech Group, Inc., Chicago, IL). The membranes were incubated in primary antibody at 4°C overnight. After incubation in primary antibody, the membranes were washed for 5 minutes in TBS-T (5x) before incubation with 1:5000 rabbit anti-mouse horseradish peroxidase conjugated secondary antibody for AMPD2 (Abcam) or 1:5000 goat anti-rabbit secondary for ADSS (EMD). Protein bands were detected using the Pierce supersignal chemiluminescence substrate (Thermoscientific) in the G:Box imaging system (Syngene, Frederick, MD). Densitometry of the protein band corresponding to AMPD2 or ADSS was obtained using

ImageJ (National Institutes of Health, Bethesda, MD) and results were normalized to that of wildtype.

*Sample preparation for PNC enzyme activity assays:* Wildtype mouse hearts and HIF-1α Non-I, 2D, and 6D mouse hearts were perfused briefly with phosphate buffer saline to wash out excess blood. Hearts were then ground into a fine powder over liquid nitrogen and homogenized in a buffer consisting of 0.089M potassium phosphate, 0.18M potassium chloride, and 0.1mM dithiothreitol with 1% v/v protease inhibitor cocktail. BCA assay was used to determine the protein concentration of the homogenates.

*AMP deaminase activity*: AMP deaminase enzyme activity was assessed using a procedure described by Raffin (8). Homogenates were diluted to a protein concentration of 2.5 µg/µl. 400 µl of the 2.5 µg/µl heart homogenate, which corresponds to 1 mg of protein, were added to 2 ml of a reaction mixture containing (mM): 50 cacodylic acid, 150 KCl, 10 AMP. A 250 µl aliquot of the reaction was taken upon initiation of the reaction ($t_0$) and after 2 hours of incubation at room temperature. 125 µl of 4% perchloric acid (Alfa Aesar, Ward Hill, MA) was used to extract nucleotides from the reaction aliquots and IMP was detected at 254 nm using the high performance liquid chromatography procedure described in chapter 2 and quantified using a calibration curve. IMP production was obtained by subtracting the initial ($t_0$) level from that detected after 2 hours of incubation. AMP deaminase specific activity was reported as nmoles of IMP produced per minute per mg protein.

*ADSS activity:* Homogenates were diluted to a protein concentration of 2 µg/µl in substrate free reaction buffer consisting of 27 mM imidazole-HCl, 50 mM potassium chloride, 8.3 mM $MgCl_2$, 5 mM $KH_2PO_4$, and 0.16 mM DTT (pH 7.2). 250 µl of the 2 µg/µl homogenate were added to 350 µl of substrate buffer (i.e. substrate free reaction buffer plus substrate). The final concentration of substrates (mM) in 600 µl of reaction volume was 0.5 IMP, 0.3 GTP, 16 creatine phosphate, 4 aspartate, and 0.25 mg creatine phosphokinase. The ADSS reaction is powered by hydrolysis of GTP and the resulting GDP strongly inhibits this reaction step in the PNC. As a result, the nucleotide triphosphate regenerating system consisting of creatine phosphate and creatine phosphokinase was added to the reaction to regenerate GTP. S-AMP levels, measured by HPLC at 268 nm were determined upon initiation of the reaction ($t_0$) and after 30 minutes ($t_{30}$) of incubation at 37$^{o}$C and quantified using a calibration curve. S-AMP production was calculated by subtracting the initial ($t_0$) S-AMP content from that found after 30 minutes ($t_{30}$) of reaction. Specific activity of ADSS was expressed as µmoles of S-AMP produced per minute per mg protein.

*ADSL activity:* The protein concentration of heart homogenates was diluted to 2 µg/µl using 50 mM tris (pH 7.4). 250 µg (125 µl) of each sample were added to 175 µl of reaction buffer (50 mM tris with 0.25 mM S-AMP). Immediately after the reaction was started ($t_0$), a 125 µl aliquot of sample was taken and nucleotides were extracted with 62.5 µl of 4% perchloric acid in order to get a basal level of AMP. The remaining sample was allowed to react for 30 minutes ($t_{30}$) at 37$^{o}$C. After the 30 minute incubation period, 125 µl of sample were drawn and nucleotide extraction was again extracted with 62.5 µl of 4% perchloric acid. Perchloric acid extracts were adjusted to pH 7 with a

buffer consisting of 4/5 volume 2M KOH and 1/5 volume 1M $KH_2PO_4$. AMP in the neutralized perchloric acid extracts was determined using reverse phase HPLC at 254 nm and quantified with its corresponding calibration curve. AMP production was calculated by subtracting the initial ($t_0$) AMP content from that detected after 30 minutes ($t_{30}$) of reaction incubation. ADSL activity is reported as μmoles of AMP generated per minute per mg protein.

*Statistical analysis:* Data reported are expressed as means±SEM. Statistical significance was tested using one-way ANOVA followed by SNK post-hoc test using GraphPad Prism 5 (La Jolla, CA). Where appropriate, Student's t-test was used to test for statistical significance.

## RESULTS

Figure 3.3a shows that mRNA levels of the enzyme regulating entry into the purine nucleotide cycle (PNC), AMP deaminase isoform 2 (AMPD2) is upregulated in Non-I, 2D, and 6D mouse hearts as compared to wildtype. It took 6 days of HIF-1α induction (6D) however, for AMPD2 protein to accumulate to levels greater than that found in wildtype mouse hearts (Figures 3.3b & 3.3c). In concurrence with the elevated protein expression of AMPD2 in the 6D mouse heart extracts, we find that activity of AMP deaminase was increased relative to wildtype hearts (Figure 3.3d). We did not observe any changes in protein or activity level of adenylosuccinate synthetase and adenylosuccinate lyase between wildtype and HIF-1α expressing (6D) mouse hearts (Figures 3.4 & 3.5).

## DISCUSSION

The purine nucleotide cycle has been shown to operate in skeletal muscle, kidney, brain, liver and heart (1, 6, 7, 9, 11). Here, we find that the enzyme regulating entry into the purine nucleotide cycle, AMP deaminase isoform 2 (AMPD2) is induced by HIF-1α. During ischemia, glycolysis is used to generate ATP. However, the rate at which ATP is produced from glycolysis cannot match the rate of ATP consumption. Thus, ATP is depleted and AMP accumulates. AMP can be metabolized to IMP by AMP deaminase. Metabolism of IMP by subsequent enzymes of the PNC regenerates AMP, which can be thought of as a conservation mechanism for the heart's adenine nucleotides. In addition to glycolysis, which supplies ATP, we now know that HIF-1α induces AMPD2, the entry point to the PNC. These two pathways may function to enhance the preservation of the heart's adenine nucleotide pool during ischemia. Taken together, these results provide the rationale for us to perform a detailed investigation on the effect that HIF-1α exerts on nucleotide metabolism in the ischemic heart, results of which will be presented in the next chapter.

**FIGURES**

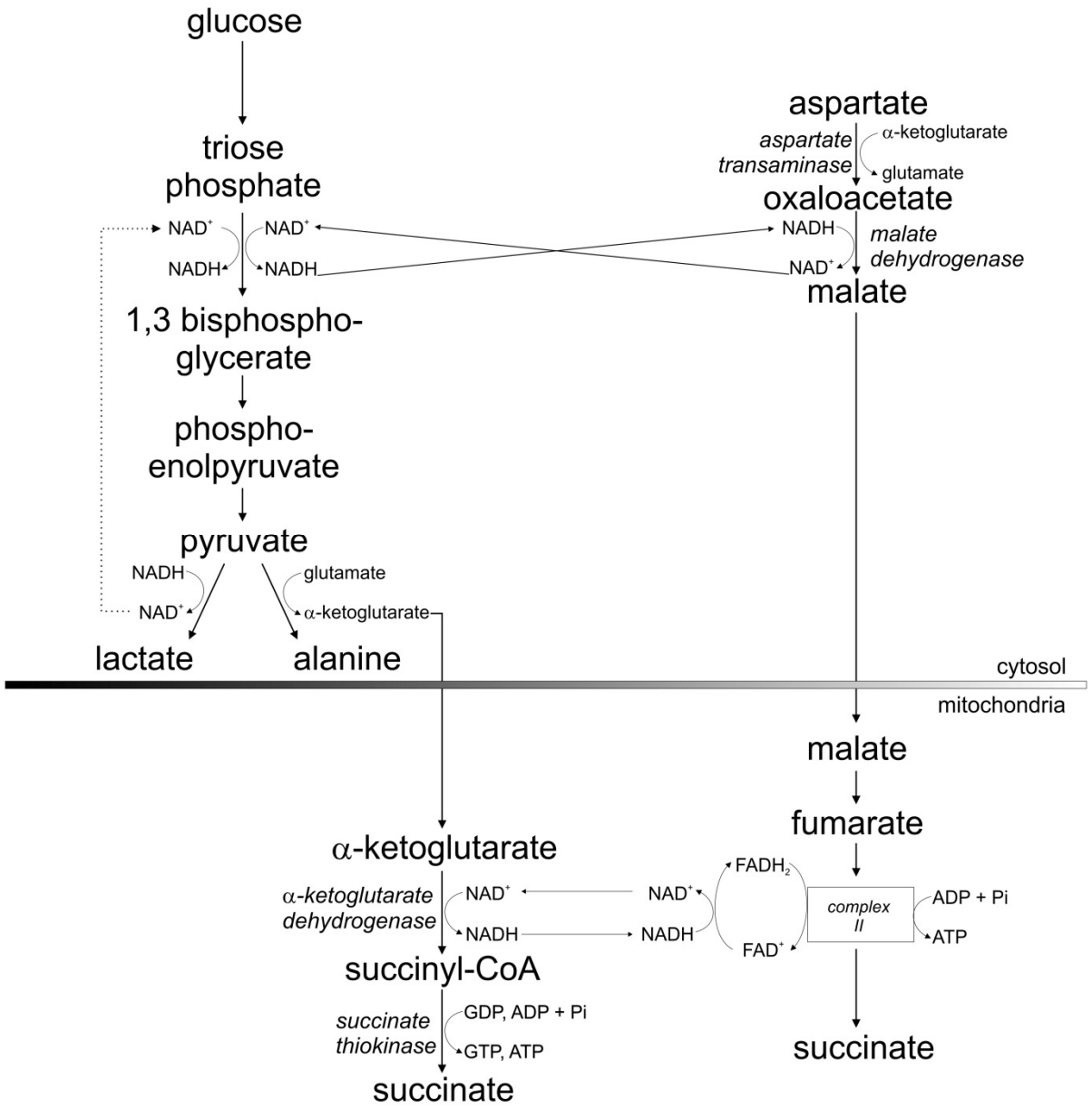


Figure 3.1: *Pathways leading to succinate production during anoxia in diving mammals as proposed by Peter Hochachka.* One pathway starts with the conversion of pyruvate to alanine, which generates α-ketoglutarate. α-Ketoglutarate then enters the mitochondria and is converted to succinyl-CoA. In a reaction catalyzed by succinate thiokinase, succinyl-CoA is converted to succinate and GTP or ATP is concamittantly produced. An alternative pathway initiates when aspartate becomes transaminated to oxaloacetate. Malate dehydrogenase then converts oxaloacetate to malate, which enters the mitochondria where it is metabolized to fumarate. Fumarate then becomes reduced to succinate at complex II. Adapted from Hochachka et al. (3)

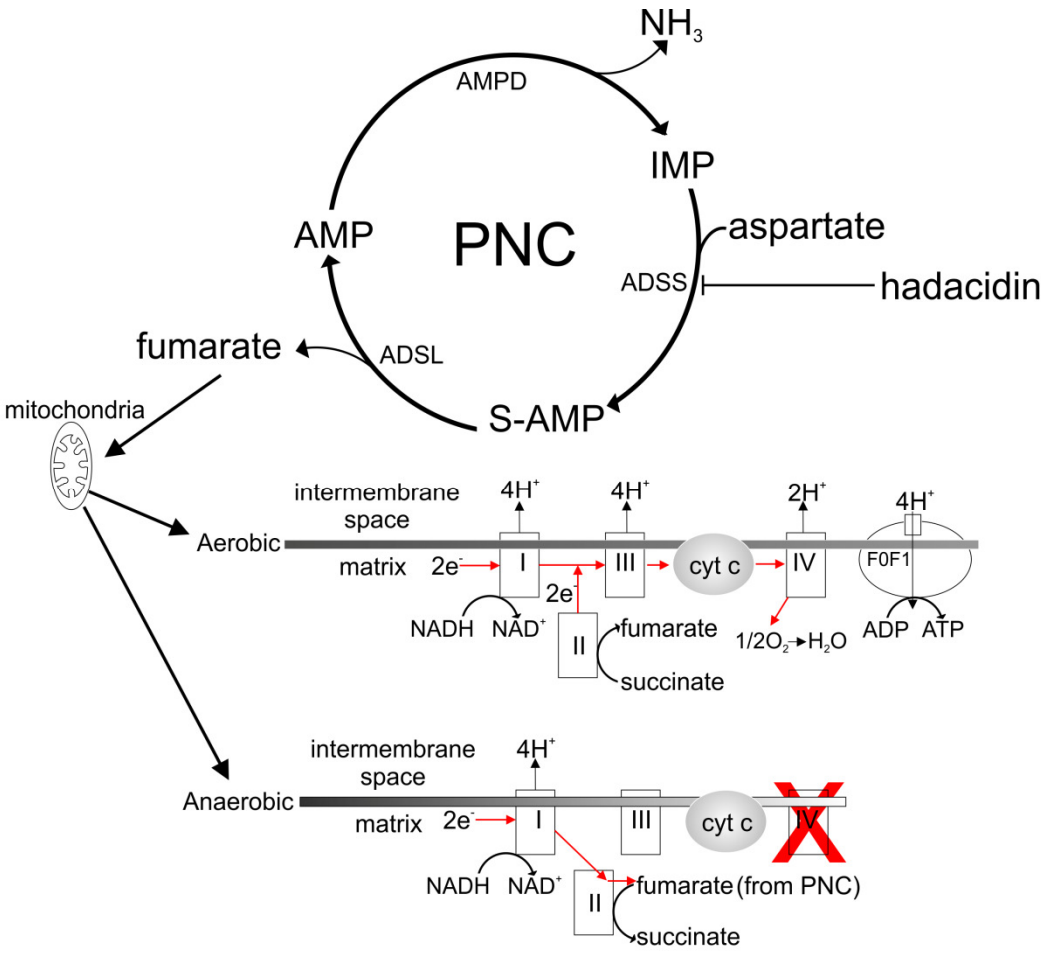


Figure 3.2: *The purine nucleotide cycle (PNC).* The PNC starts when AMP is deaminated to IMP by AMP deaminase (AMPD). Subsequently, IMP is converted to S-AMP in a reaction catalyzed by adenylosuccinate synthetase (ADSS). S-AMP is converted back to AMP utilizing adenylosuccinate lyase (ADSL). The ADSL reaction step also produces fumarate, which can be used as an alternative terminal electron acceptor in the electron transport chain to maintain anaerobic mitochondrial respiration.

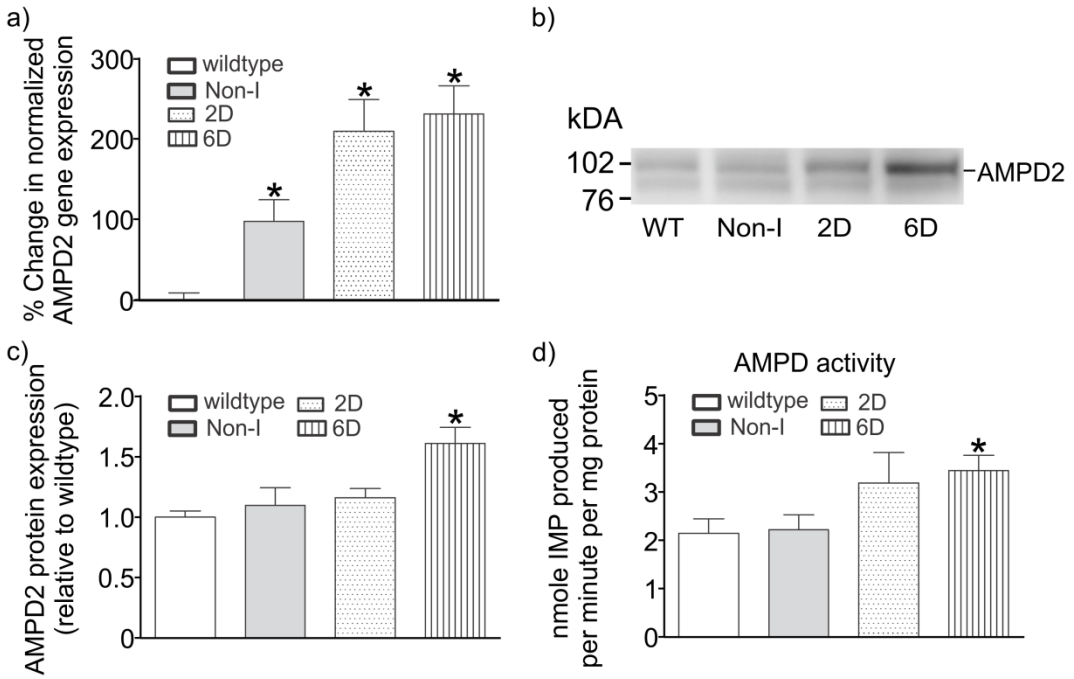


Figure 3.3: *HIF-1α upregulates the mRNA, protein, and activity of AMP deaminase (AMPD) in mouse hearts.* Hearts were obtained from wildtype mice and those in which the HIF-1α-PPN transgene was suppressed (Non-I) or allowed to be expressed for 2 days (2D) or 6 days (6D). a) AMPD2 gene expression in mouse heart homogenates was examined using qPCR and normalized to reference gene transferrin. Results are expressed as the % change in gene expression relative to WT (n=6). b) Western blot showing AMPD2 protein expression in mouse heart homogenates. c) Quantification of AMPD2 protein levels in mouse heart homogenates (n=6-8). d) AMPD activity assessed by the amount of IMP produced per minute per mg protein in a buffer system containing excess AMP (n=5). * P<0.05 versus WT.

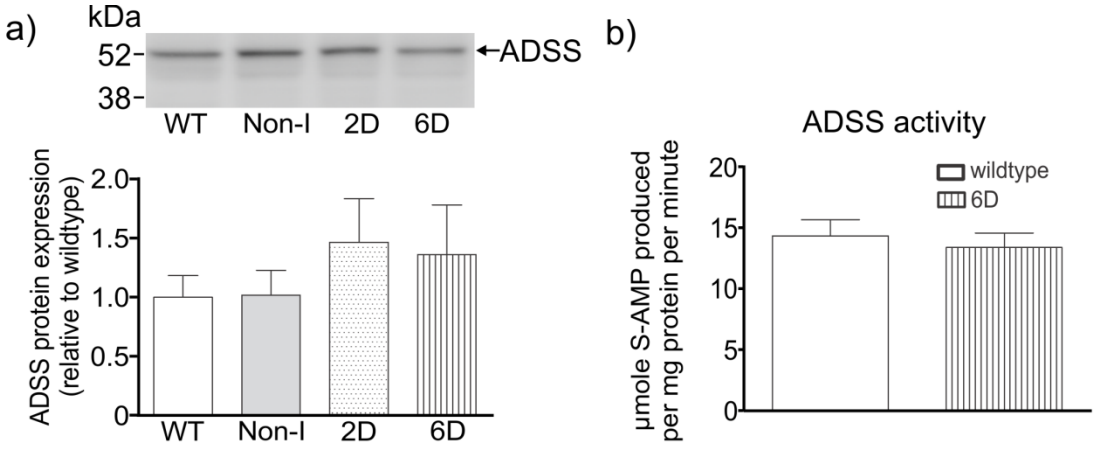


Figure 3.4: *HIF-1α does not change the protein expression or activity of ADSS in the heart*. Extracts were obtained from wildtype (WT) mouse hearts and those where the HIF-1α-PPN

transgene has been suppressed (Non-I), expressed for 2 days (2D), or expressed for 6 days (6D) to examine protein and activity levels of ADSS. a) Representative western blot showing ADSS expression in adult mouse heart extracts (top); average ADSS protein expression in mouse hearts (n=3-5) (bottom). b) Average ADSS activity in mouse hearts (n=5).

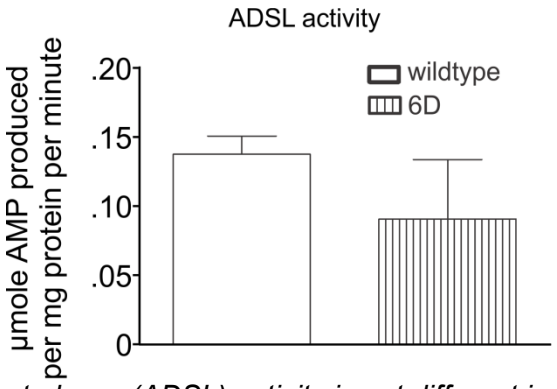


Figure 3.5: *Adenylosuccinate lyase (ADSL) activity is not different in wildtype and HIF-1α expressing 6 day (6D) mouse hearts*. Hearts were obtained from wildtype mice and those that have been denied doxycycline to express the HIF-1α-PPN transgene for 6 days (6D) and activity of adenylosuccinate lyase was examined. n=3.

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# CHAPTER 4

## HIF-1α IN THE HEART: REMODELING OF NUCLEOTIDE METABOLISM LEADING TO ATTENUATION OF ADENOSINE ACCUMULATION DURING ISCHEMIC STRESS


## SUMMARY

$O_2$ delivery to heart tissue is compromised during ischemia. As a result, ATP production via oxidative phosphorylation becomes inhibited. While glycolysis can produce ATP anaerobically, the rate of ATP synthesis from this process cannot match the rate of ATP consumption. Therefore, ischemia in the heart leads to a net depletion of ATP and the nucleotide pool while nucleobases accumulate. Having identified that HIF-1α upregulates AMP deaminase, the entry point to the adenylate conserving purine nucleotide cycle (PNC), we sought to examine how HIF-1α influences nucleotide metabolism in the ischemic heart. Here, we found that HIF-1α expression in the heart prevents the degradation of nucleotides under the ischemic setting. As ATP degrades during ischemia, AMP accumulates. AMP can be metabolized to adenosine by 5'-nucleotidase isoform 1 or the PNC. Here, we noted that HIF-1α limits adenosine accumulation in the ischemic heart, consistent with the shunting of AMP metabolism towards the cardioprotective PNC. Our group previously showed that HIF-1α confers ischemic tolerance by allowing cardiomyocytes to use PNC derived fumarate to sustain anaerobic mitochondrial respiration. Upon further examination, we found that HIF-1α also upregulates adenosine deaminase, an enzyme that degrades adenosine. HIF-1α's role in reducing adenosine accumulation in the ischemic heart may seem paradoxical since adenosine is known to be a cardioprotective molecule. However, recent evidence indicates that chronic exposure to adenosine can cause toxicity. Therefore, HIF-1α expression may allow the heart to circumvent the toxicity caused by chronic adenosine

exposure. Collectively, we found that HIF-1α not only preserves the hearts adenine nucleotide pool, but it also upregulates pathways whose role in cardioprotection may go beyond the remodeling of nucleotide metabolism.

## INTRODUCTION

During ischemia, ATP production via oxidative phosphorylation ceases. Glycolysis can generate ATP anaerobically, however, the rate of ATP synthesis from glycolysis cannot match the rate at which ATP is consumed. ATP and the heart's nucleotide pool becomes rapidly depleted during ischemia (Figure 4.1) (1, 15, 16, 34). This results in the formation of non-phosphorylated nucleoside products such as inosine and adenosine, which can freely diffuse into the extracellular space, where they are further degraded to nucleobases such as hypoxanthine (Figure 4.1). The loss of nucleosides and nucleobases to the extracellular space reduces the amount of precursors that are available for re-synthesis of purine nucleotides via the salvage pathway. Thus, this represents a critical route in which the cellular nucleotide pool is depleted during ischemic insult.

Maintenance of the adenine nucleotide pool is of great importance in the ischemic heart, as these are the metabolites necessary for ATP production. The synthesis of adenine nucleotides from *de novo* pathways is very slow and energetically expensive (25) and the loss of precursors limits nucleotide resynthesis from salvage pathways. Furthermore, *de novo* nucleotide synthesis requires glucose-6-phosphate, an intermediate in glycolysis (see review by Lunt et al, 2011) (20). Under the ischemic setting, not only is oxygen delivery diminished, but compromised blood flow in the

coronary vasculature limits the delivery of nutrients such as glucose, which is metabolized by glycolysis to generate glucose-6-phosphate. As such, the diminished glucose delivery to the heart tissue further hinders *de novo* nucleotide synthesis. While contractile activity is abolished upon the onset of ischemia, the heart still relies on ATP to maintain ionic homeostasis. Hence, mechanisms that help conserve the heart's valuable nucleotide resources could prove protective.

In the previous chapter, we show that the reaction step regulating entry into the purine nucleotide cycle (PNC), AMP deaminase isoform 2 (AMPD2) is induced by HIF-1α. Activity of the PNC has been demonstrated in isolated cardiomyocytes subjected to anoxic stress (13, 14, 33). Sabina et al. proposed that the PNC acts to conserve the cellular adenine nucleotide pool (32). As ATP is degraded during ischemia, AMP accumulates. The fate of AMP is either metabolism to adenosine by 5'-nucleotidase isoform 1 (5'NT-I) or IMP by AMPD2 (Figure 4.1). Adenosine is uncharged and freely diffuses out of the myocyte. Alternatively, the IMP that is generated by AMP deaminase can be utilized by subsequent steps of the PNC to regenerate AMP, thus fulfilling the PNC's role in adenine nucleotide conservation. In addition to glycolysis, we have identified another HIF-1α induced pathway that may exert protective effects on the heart's nucleotide resources during ischemia. As such, it became important for us to examine in detail, the effects that HIF-1α exerts on nucleotide metabolism in the heart during ischemic stress.

## EXPERIMENTAL PROCEDURES

*Nucleotide metabolism:* Nucleotide metabolism during ischemia was examined in WT as well as HIF-1α Non-I, 2D, and 6D mouse hearts. All hearts were perfused with Krebs buffer containing (in mM): 118.5 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 24.8 $NaHCO_3$, 2.5 $CaCl_2$, and 10.6 glucose on the Langendorff apparatus for 25 minutes to allow stabilization. Following the stabilization period, hearts were subjected to either 5, 10, 20, or 30 minutes of ischemia, or perfusion for 2.5 minutes with glucose-free Krebs buffer containing 5 mM glycolytic inhibitor iodoacetate (Acros Organics, Morris Plains, NJ) followed by 20 minutes of ischemia. After ischemic stress, nucleotides were extracted with 400 µl of 4% perchloric acid and were measured with HPLC as detailed in chapter 2. Nucleotides were quantified using their respective calibration curve and results were expressed as µmoles of nucleotide per gram dry tissue.

*Protein expression of adenosine deaminase:* Protein samples from mouse hearts were prepared according to experimental procedures outlined in chapter 2. Adenosine deaminase was probed using a rabbit polyclonal antibody diluted 1:400 in TBS-T (Novus Biologicals, Littleton,CO) and incubated overnight with rocking at 4$^{o}$C. After incubation in primary antibody, the membranes were washed 5x5 minutes with TBS-T and then incubated with a goat anti-rabbit horseradish peroxidase conjugated secondary antibody (EMD) for 1 hour at room temperature. The band corresponding to adenosine deaminase was detected using the Pierce supersignal chemiluminescence substrate in the G:BOX imaging system (Syngene, Frederick, MD). Densitometry of the protein band corresponding to adenosine deaminase was done using ImageJ (National Institutes of Health, Bethesda, MD) and results were normalized to that of wildtype.

*Adenosine deaminase activity:* Activity of adenosine deaminase was determined in WT and 6D mouse heart homogenates according to a method developed by Hartwick et al (11). The volume of homogenate corresponding to 250 µg protein was added to a reaction mixture containing 0.065F $KH_2PO_4$, 0.06F $Na_2HPO_4$, and 17.5 mM adenosine. The volume of reaction mixture added to the homogenates was such that the final adenosine concentration in the reaction was 3.5 mM. Half of the reaction was immediately taken out to obtain the initial inosine content ($t_0$). The remaining half of the reaction was allowed to incubate at room temperature for 40 minutes. HPLC was used to monitor the amount of product (i.e. inosine) formed during the reaction. The amount of inosine produced during the incubation period is obtained by subtracting the inosine content at the start of the reaction ($t_0$) from the inosine content after the 40 minute incubation period. Adenosine deaminase specific activity was expressed as nmoles of inosine formed per minute per mg protein.

*Statistical analysis:* Data reported are expressed as means±SEM. Statistical significance was tested using one-way ANOVA followed by SNK post-hoc test using GraphPad Prism 5 (La Jolla, CA). Where appropriate, Student's t-test was used to test for statistical significance.

## RESULTS

Figure 4.2 shows that HIF-1α allows for better preservation of the heart's total adenine nucleotide pool (i.e. sum of ATP, ADP, and AMP) during ischemic stress. As ATP degrades during ischemia, AMP accumulates. AMP accumulation was hastened in wildtype mouse hearts during ischemia (Figure 4.3). The accumulation of

AMP during ischemia was delayed in the HIF-1α-PPN mouse hearts. It took 20 minutes of ischemic stress for AMP in Non-I and 2D hearts to reach levels equivalent to wildtype (Figure 4.3). After 30 minutes of ischemia, AMP accumulation was equal across the heart groups (Figure 4.3).

AMP that accumulates during ischemia has two fates. First, AMP can be degraded to adenosine via a reaction catalyzed by 5'-nucleotidase (Figure 4.1). As evident by Figure 4.4a, adenosine accumulated readily in wildtype hearts upon exposure to ischemic insult. On the other hand, adenosine accumulation in Non-I and 2D hearts was delayed and did not match the levels found in wildtype until 30 minutes of ischemia have elapsed. In hearts that have been expressing HIF-1α for 6 days, adenosine levels failed to increase even after 30 minutes of ischemia (Figure 4.4a). Alternatively, AMP can be metabolized to IMP during ischemia. The Non-I, 2D, and 6D heart accumulated lower levels of IMP as compared to wildtype after 20 and 30 minutes of ischemia (Figure 4.4b).

Figure 4.5a shows that inosine levels during the initial 20 minutes of ischemia was lower in 6D hearts as compared to wildtype, Non-I, and 2D hearts. Nucleoside phosphorylase can further metabolize inosine to hypoxanthine, which is further degraded to xanthine. Here, we found that while hypoxanthine and xanthine levels increased in the ischemic heart, they were not different in HIF-1α-PPN hearts as compared to wildtype at any time point of ischemia (Figures 4.5b & 4.5c).

In this initial series of studies, we found that expression of HIF-1α for 6 days prevented the buildup of adenosine in the ischemic heart. In chapter 2, we showed that

HIF-1α expression for 6 days enhances glycolytic activity and preservation of ATP in the heart during ischemia. Here, we also showed that in hearts where HIF-1α has been expressed for 6 days, the accumulation of AMP was lower during the initial 20 minutes of ischemia (Figure 4.3). Thus, the reduction of adenosine accumulation in HIF-1α expressing hearts may merely reflect the lower amount of AMP available for metabolism by 5'-nucleotidase.

To test our hypothesis, we perfused hearts with 5 mM of glycolytic inhibitor iodoacetate followed by 20 minutes of ischemia. At the end of this ischemic stress protocol, ATP, ADP, and AMP were reduced to similar levels in wildtype and 6D hearts (Figure 4.6). Yet, 6D hearts still failed to accumulate adenosine to levels similar to that in wildtype, while IMP level was not different between these two heart groups (Figure 4.7). Here, it is crucial to note that inosine, hypoxanthine, and xanthine accumulated to higher levels in 6D mouse hearts as compared to wildtype after this ischemic stress protocol (Figure 4.7). Taken together, our results indicate that pathways besides glycolysis must be responsible for decreasing the accumulation of adenosine in the ischemic 6D hearts.

An obvious explanation for the attenuation of adenosine in 6D hearts is the increased expression of AMP deaminase isoform 2 (AMPD2). However, we also considered the following two factors in our endeavor to identify mechanisms that attenuated adenosine levels in the ischemic 6D HIF-1α expressing hearts. First is the observation that less adenine nucleotides were degraded in the 6D hearts during the ischemic time course but levels of hypoxanthine and xanthine that accumulated was

equivalent to wildtype. Second, ATP, ADP, and AMP were depleted to the same level in wildtype and 6D hearts when glycolysis was blocked prior to ischemia but yet 6D hearts were able to accumulate more inosine, hypoxanthine, and xanthine, the downstream degradation products of adenosine and IMP (Figure 4.1). These two observations led us to believe that adenosine deaminase was upregulated in 6D hearts, thus allowing for more rapid degradation of adenosine (Figure 4.1). Figure 4.8 shows that indeed, protein and activity of adenosine deaminase is induced in the extracts of 6 day HIF-1α expressing hearts.

## DISCUSSION

In these studies, we find that HIF-1α preserves adenine nucleotides during ischemia in the heart. The accumulation of nucleotide breakdown products required the shortest duration of ischemic stress in wildtype mouse hearts. A delay is observed in Non-I and 2D hearts. The breakdown of adenine nucleotides is slowest in the 6D hearts. These results suggests a pseuo-dose response such that nucleotide depletion during ischemia is fastest in wildtype, slower in Non-I and 2D hearts, and slowest in 6D hearts.

Studies where we blocked glycolysis prior to ischemia indicated additional mechanisms contribute to the reduction of adenosine accumulation that we have observed during ischemia in 6 day HIF-1α expressing hearts. The first mechanism we considered was the reaction catalyzed by adenosine kinase, which converts adenosine back to AMP. However, as ATP is needed as a phosphate donor to adenosine, the likelihood that adenosine kinase is functional under ischemic conditions is slim. Indeed,

it has been shown that adenosine kinase activity is suppressed during ischemic stress (5, 21, 27).

Our finding that HIF-1α induces the expression of AMP deaminase isoform 2 (AMPD2) provides one explanation to our observation that HIF-1α expression reduces adenosine accumulation in ischemic heart. This suggests that AMP metabolism is diverted towards the PNC during ischemia in hearts that express HIF-1α, which should enhance the production of fumarate. As our group has shown in the past, HIF-1α allows cardiomyocytes to utilize fumarate derived from the PNC to maintain anaerobic mitochondrial respiration, thus leading to enhanced ischemic tolerance. Our observation that adenosine accumulation is limited in HIF-1α expressing hearts is consistent with the higher activity of the cardioprotective PNC. Surprisingly, we observed that in HIF-1α expressing hearts, IMP did not accumulate to levels higher than wildtype hearts during ischemia, even though HIF-1α upregulates AMP deaminase. Unlike tetanic activity of skeletal muscle, where IMP accumulation is proportional to the amount of ATP degraded (26), IMP has not been detected in ischemic hearts (16). This has led Manfredi to propose that unknown mechanisms are preventing IMP accumulation in the heart during ischemia (24). Taken together, a multitude of factors may play a role in determing the levels of IMP in the heart during ischemic stress and we hope to identify these in future studies.

To further identify pathways that contribute to reduced adenosine accumulation during ischemia, we focused on our observation that 6D HIF-1α expressing hearts were able to achieve equal or higher levels of adenosine breakdown products during

ischemia as compared to wildtype mouse hearts. Consistent with this result, we found that adenosine deaminase protein and activity in heart extracts from 6D mice were higher than wildtype. Our results are consistent with work done by Eltzschig, which showed that hypoxia induces the mRNA and protein expression of adenosine deaminase (7).

The finding that HIF-1α limits adenosine accumulation contradicts the protective role that this metabolite has been ascribed, particularly in the realm of cardioprotection (17, 29, 35, 37). This is achieved through signaling via various isoforms of cell surface adenosine receptors (6, 19, 28). Immune response has been recognized to contribute to the adverse effects associated with ischemia (10, 18). Various studies show that adenosine plays a role in suppressing immune cells by inhibiting their transmigration through the vasculature and reducing their production of proinflammatory cytokines (3, 9, 12). On the other hand, it has been recognized that chronic exposure to adenosine causes toxicity. Blackburn has postulated that toxicity from chronic adenosine exposure may arise from receptor downregulation and activation of adenosine receptors on surrounding immune cells (2). Downey's group showed that prolong exposure of adenosine receptors to agonist led to abolishment of cardioprotection provided by preconditioning (36). These results are suggestive of receptor downregulation as a result of chronic adenosine exposure. Adenosine has been shown to stimulate mast cell degranulation (31). In addition, adenosine may also be proarrhythmic (8, 23). Furthermore, adenosine has been shown to induce fibrosis (4, 22, 30). Thus, HIF-1α may play a role in maintaining the intricate balance between the benefits of adenosine signaling and ill-effects associated with chronic adenosine exposure. Future studies

should examine whether adenosine exposure, long-term, triggers maladaptive responses during ischemia and whether HIF-1α provides an approach to limit this.

Collectively, nucleotide metabolism is remodeled by HIF-1α. These remodeling processes result in better preservation of adenine nucleotides during ischemia. More importantly, HIF-1α reduces the amount of adenosine that accumulates in the ischemic heart. This result is consistent with higher activity of the cardioprotective PNC pathway and also suggests that HIF-1α expression in the heart may limit the toxicity associated with chronic exposure to adenosine.

**FIGURES**

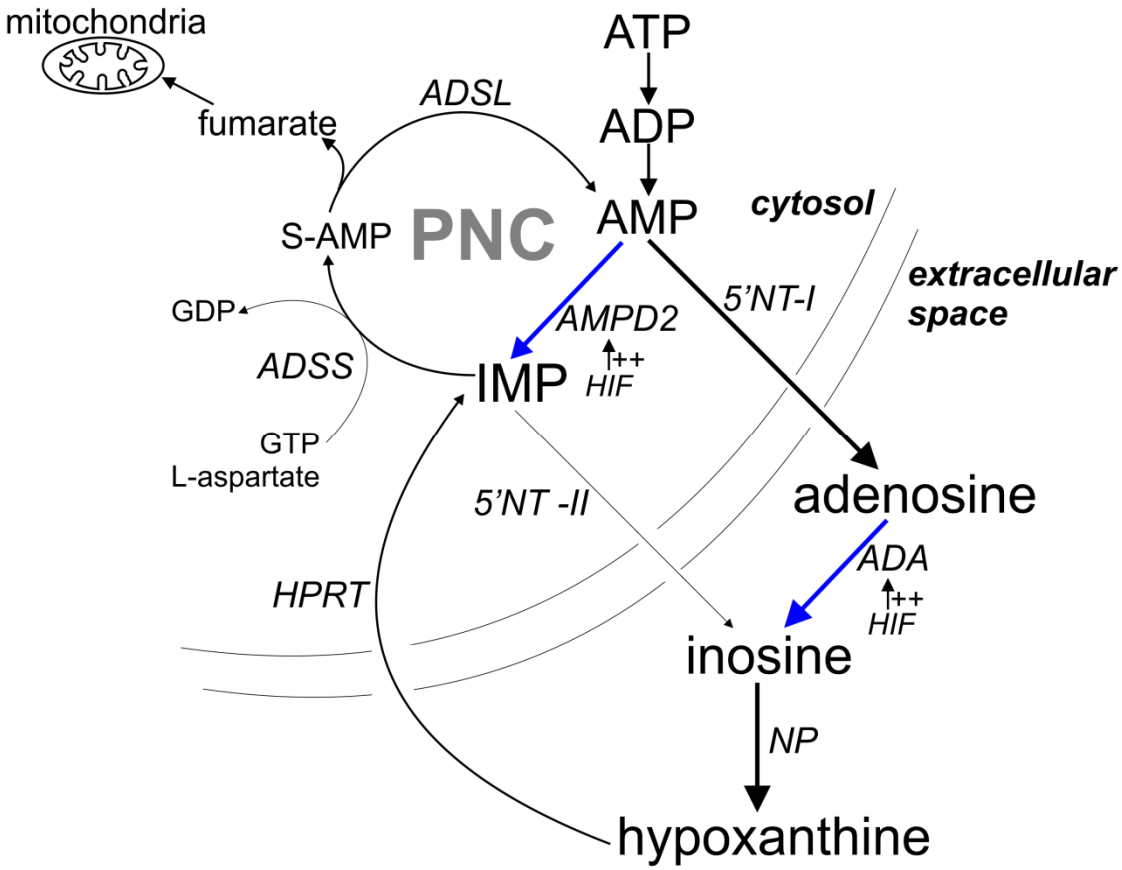


Figure 4.1: *Nucleotide degradation pathways in the ischemic heart*. During ischemia, the rate of ATP consumption exceeds its synthesis from glycolysis. As a result, there is a net degradation of ATP and the heart's nucleotide pool while the nucleobase hypoxanthine accumulates. PNC = purine nucleotide cycle. AMPD2 = AMP deaminase isoform 2. ADSS = adenylosuccinate synthetase. ADSL = adenylosuccinate lyase. 5'NT-1 = 5'-nucleotidase isoform 1 (AMP specific). 5'NT-II = 5'-nucleotidase isoform 2 (IMP specific). HPRT = hypoxanthine phosphoribosyl transferase. HIF induced pathways are denoted by blue arrows. Major pathways that have been proposed previously by others are shown in bold black arrows. Adapted from Jennings et al. (16).

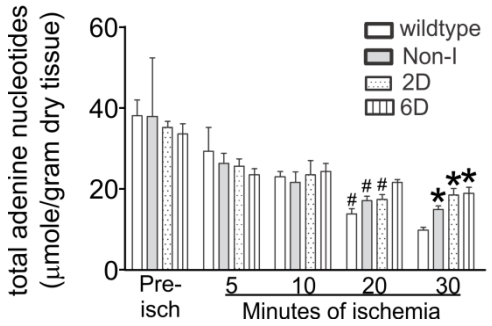


Figure 4.2: *Wildtype mouse hearts are inept at preserving the adenine nucleotide pool during ischemia*. Wildtype mouse hearts, hearts not expressing the HIF-1α-PPN transgene (Non-I), and hearts made to express the HIF-1α-PPN transgene for 2 days (2D) and 6 days (6D) were subjected to the indicated durations of ischemia. Nucleotides were measured using HPLC and the sum of ATP, ADP, and AMP was taken as the total adenine nucleotide pool. Pre-isch denotes pre-ischemia. * P<0.05 versus wildtype. # P<0.05 versus 6D. n=3-9.

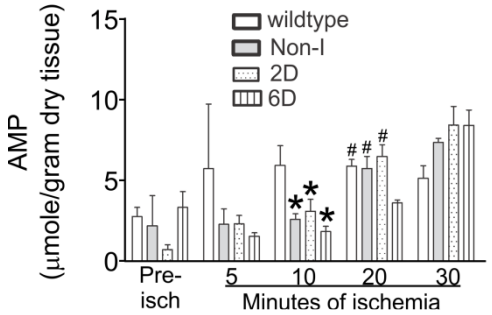


Figure 4.3: *AMP levels in ischemic adult mouse hearts*. Hearts were obtained from wildtype mice and those in which the HIF-1α-PPN transgene is suppressed (Non-I), expressed for 2 days (2D) or 6 days (6D). AMP levels were measured at the indicated durations of ischemic stress. Pre-isch denotes pre-ischemia. * P<0.05 versus wildtype. # P<0.05 versus 6D. n=3-9.

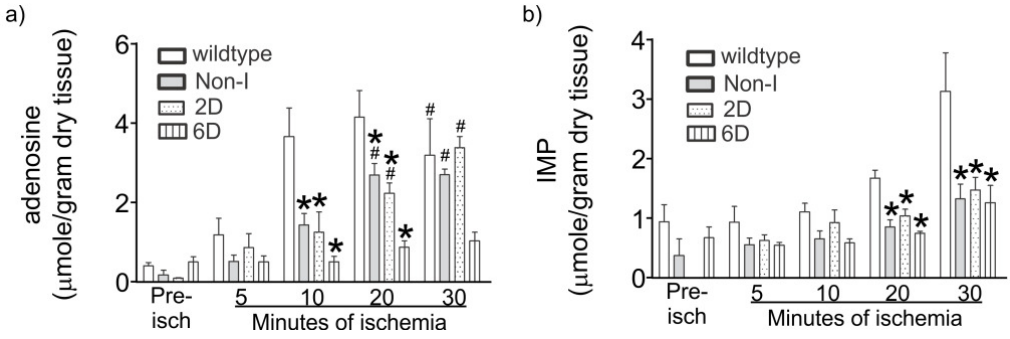


Figure 4.4: *HIF-1α reduces the amount of adenosine and IMP that accumulates in the ischemic heart.* Wildtype mouse hearts as well as those not expressing the HIF-1α-PPN transgene (Non-I), expressing the HIF-1α-PPN transgene for 2 days (2D) and 6 days (6D) were subjected to the

indicated durations of ischemia and nucleotides were measured with HPLC. a) Adenosine. b) IMP. * P<0.05 versus wildtype. # P<0.05 versus 6D. n=3-9.

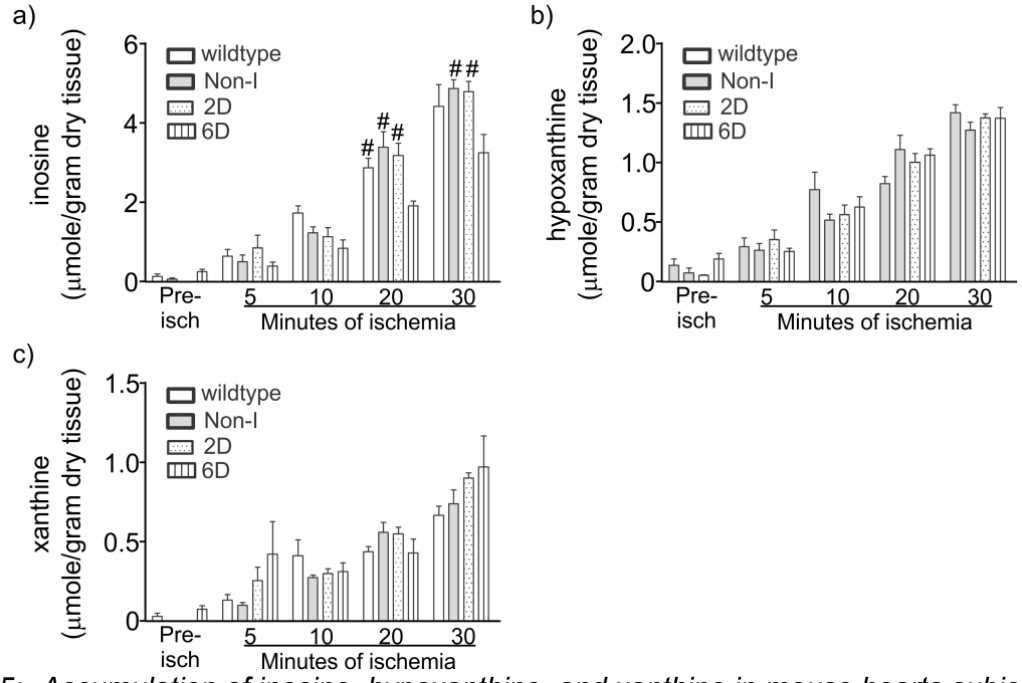


Figure 4.5: *Accumulation of inosine, hypoxanthine, and xanthine in mouse hearts subjected to total ex vivo ischemic stress*. Hearts were obtained from wildtype mice and those in which the HIF-1α-PPN transgene is suppressed (Non-I), expressed for 2 days (2D) or 6 days (6D). Hearts were subjected to the indicated durations of ischemia. Nucleotides were extracted and measured using HPLC. Pre-isch denotes pre-ischemia. a) Inosine. b) Hypoxanthine. c) Xanthine. * P<0.05 versus wildtype, # P<0.05 versus 6D. n=3-9.

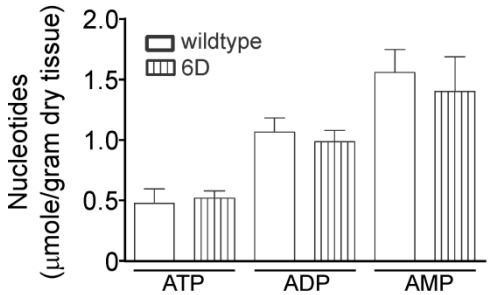


Figure 4.6: *Inhibition of glycolysis reduces post-ischemic content of ATP, ADP, and AMP to similar levels in wildtype hearts and those expressing the HIF-1α-PPN transgene for 6 days*. Hearts were obtained from wildtype mice and those in which the HIF-1α-PPN transgene was expressed for 6 days (6D). Hearts were perfused for 2.5 minutes with 5 mM glycolytic inhibitor

iodoacetate then subjected to 20 minutes of *ex vivo* ischemia. After ischemic stress, nucleotides were extracted and measured using HPLC. n=4-5.

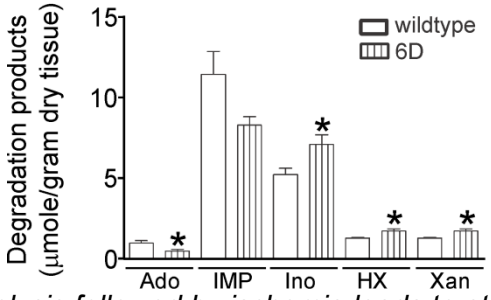


Figure 4.7: *Inhibition of glycolysis followed by ischemia leads to attenuation of adenosine (Ado) accumulation but increases in accumulation of inosine (Ino), hypoxanthine (HX), and xanthine (Xan) in hearts expressing the HIF-1α-PPN transgene*. Hearts were obtained from wildtype mice and those in which the HIF-1α-PPN transgene was expressed for 6 days (6D). Hearts were perfused for 2.5 minutes with 5 mM glycolytic inhibitor iodoacetate then subjected to 20 minutes of *ex vivo* ischemia. After ischemic stress, nucleotides were extracted and measured using HPLC. * P<0.05 versus wildtype. n=4-5.

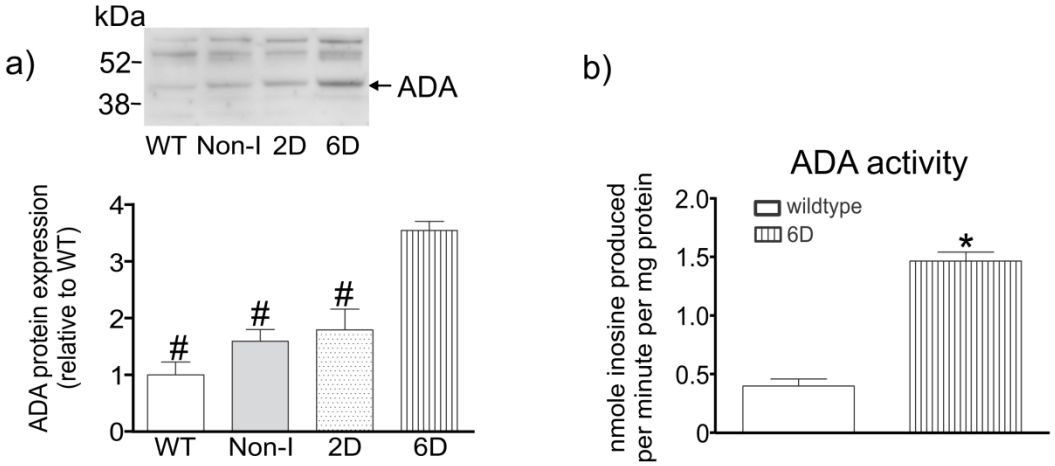


Figure 4.8: *HIF-1α expression induces the protein and activity of adenosine deaminase (ADA)*. Heart homogenates were obtained from wildtype mice and those in which the HIF-1α-PPN transgene was suppressed (Non-I), expressed for 2 days (2D), or expressed for 6 days (6D). a) Representative Western Blot showing adenosine deaminase expression (top); average expression of adenosine deaminase from 4 to 7 mouse hearts (bottom). b) Adenosine deaminase specific activity in homogenates from 5 mouse hearts. # P<0.05 versus 6D. * P<0.05 versus wildtype.

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# CHAPTER 5

## THE PURINE NUCLEOTIDE CYCLE AND ITS EFFECTS ON NUCLEOTIDE METABOLISM IN THE ISCHEMIC HEART


## SUMMARY

We found that HIF-1α reduces the accumulation of adenosine in the ischemic heart. This is consistent with the shunting of AMP metabolism towards AMP deaminase, which is upregulated by HIF-1α. AMP deaminase represents the step regulating entry into the nucleotide metabolic pathway known as the purine nucleotide cycle (PNC). To establish a better understanding of the role that the PNC plays in the ischemic heart, we perfused wildtype and 6 day HIF-1α induced (6D) mouse hearts with 50 μg/ml (0.42 mM) adenylosuccinate synthetase inhibitor, hadacidin followed by treatment of hearts to 30 minutes of *ex vivo* ischemic stress. We found that inhibition of the PNC led to a reduction of ATP, ATP:ADP, and adenylate energy charge in wildtype and 6D hearts during ischemia. The inhibition of adenylosuccinate synthetase by hadacidin should decrease the availability of fumarate used to maintain anaerobic mitochondrial respiration. Our studies allow us to propose a new physiological role for the PNC, which is that the PNC preserves ATP, ATP:ADP, and adenylate energy charge via the provision of fumarate, which is used as an alternative terminal electron acceptor in place of $O_2$ to maintain mitochondrial polarization during ischemia. This prevents the reverse mode operation of the $F_0F_1$ ATP synthase, which consumes ATP in order to maintain the mitochondrial membrane potential during ischemia. Therefore, the induction of the PNC may contribute to the better preservation of energy charge up to 20 minutes of ischemic stress that we have observed in 6D HIF-1α expressing hearts.

## INTRODUCTION

In chapter 2, we showed that HIF-1α upregulates AMP deaminase, the enzyme regulating entry into the PNC. Furthermore, our finding that HIF-1α attenuates adenosine accumulation in ischemic hearts is consistent with the induction of AMP deaminase. Studies related to the PNC have for the most part been done on skeletal muscle. On the other hand, knowledge regarding the PNC and its function in the heart remains much more elusive. Unlike skeletal muscle, studies by Taegtmeyer found that workload does not affect the activity of the PNC in heart (8). This led the group to discount any significant role for the PNC in the heart. However, the heart is particularly well equipped to maintain its high energy phosphate content as workload is varied (5). Therefore, the model system used by Taegtmeyer may not have been able to capture the physiological significance of the PNC in the heart (8). To delineate the role of the PNC in the heart, we subjected hearts to *ex vivo* ischemia, a condition similar to exercise in skeletal muscle where ATP becomes readily depleted. Hearts were subjected to 30 minutes of ischemia alone or pre-treatment with hadacidin, which is a PNC inhibitor at the step of adenylosuccinate synthetase, followed by exposure to 30 minutes of ischemic stress. Nucleotide content in the heart was measured after ischemic stress. Results were compared between wildtype and HIF-1α expressing mouse hearts.

## EXPERIMENTAL PROCEDURES

*Nucleotide metabolism:* Wildtype mouse hearts and hearts derived from our HIF-1α-PPN non-induced (Non-I), 2 day induced (2D), and 6 day induced (6D) mice were perfused with Krebs buffer containing (in mM): 118.5 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2

$KH_2PO_4$, 24.8 $NaHCO_3$, 2.5 $CaCl_2$, and 10.6 glucose on the Langendorff apparatus for 25 minutes to allow stabilization. Following the stabilization period, hearts were either subjected to 5, 10, 20, or 30 minutes of ischemia or perfused for 15 minutes with Krebs buffer containing 50 µg/ml hadacidin followed by 30 minutes of ischemia. Hearts were freeze clamped and nucleotides were extracted with 400 µl of 4% perchloric acid. The nucleotide extracts were incubated on ice for 20 minutes. Following incubation, the nucleotide extracts were centrifuged at 15,000 *g* for 15 minutes at 4°C and the supernatant containing nucleotides was removed. The remaining pellet was lyophilized in order to obtain the dry weight (grams) of tissue collected. Nucleotides were measured with HPLC and quantified with their respective calibration curve. Results are expressed as µmoles of nucleotide per gram dry tissue.

*Energy status of the heart:* The heart's energy status provides an indication of the energy that can be harnessed during ATP hydrolysis that can be used to carry out cellular work and is commonly assessed by calculating the ATP:ADP ratio. The higher the ATP:ADP ratio, the more energy is harnessed upon removal of a phosphate group from ATP. However, under conditions when cellular energetics is compromised, AMP content is much more easily perturbed compared to the content of ADP (3). For example, AMP:ATP is equal to $(ADP:ATP)^2$ (3). Thus, a 5 fold increase in ADP:ATP leads to a 25 fold amplification in AMP:ATP. Therefore, AMP is the best suited metabolite used to regulate cellular energetics. In fact, the cell utilizes AMP activated protein kinase as a regulator of cellular energetics via modulation of various metabolic pathways (4). Thus, AMP content must be accounted for when quantifying the energy status of the cell. The adenylate energy charge (EC), first proposed by Atkinson (1), is

calculated from the values of the adenine nucleotide pool and is given by Equation 1. This parameter is starting to be much more commonly used to describe cellular energetics. EC ranges from 0 to 1 and is approximately 0.8 in a healthy cell. A higher EC indicates that ATP utilization is favored.

$$\text{energy charge} = \frac{\text{ATP}+0.5\text{ADP}}{\text{ATP}+\text{ADP}+\text{AMP}} \quad \text{Equation 1}$$

*Statistical analysis:* Data reported here are expressed as means±SEM. Statistical significance was tested using one way ANOVA with SNK post-hoc test or Student's t-test in GraphPad Prism 5 (La Jolla, CA).

## RESULTS

In these studies, we observed that HIF-1α induction for 6 days (6D) provides strong preservation of ATP:ADP (Figure 2.5) and adenylate energy charge (Figure 5.1). We find that hadacidin treatment prior to ischemia led to a drastic reduction in ATP and ADP in both wildtype and 6D mouse hearts (Figure 5.2a and 5.2b). AMP levels increased in both wildtype and 6D hearts when hadacidin was administered prior to 30 minutes of ischemia (Figure 5.2c). The changes in adenine nucleotide content resulted in a substantial decrease in the heart's ATP:ADP and adenylate energy charge (Figure 5.3).

Next, we examined the effect that hadacidin had on nucleotide breakdown products adenosine and IMP in the ischemic heart. While adenosine levels did not change in 6D hearts, it more than doubled in hearts from wildtype mice (Figure 5.4a). We find that hadacidin had no effect on IMP content in wildtype hearts and 6D hearts that were subjected to 30 minutes of *ex vivo* ischemia (Figure 5.4b). Adenosine and

IMP can both be degraded to inosine, which is then metabolized by nucleoside phosphorylase to hypoxanthine. We noted that levels of these metabolites were unaltered when hadacidin was administered prior to the initiation of ischemic stress (Figure 5.5).

## DISCUSSION

The purine nucleotide cycle has been postulated to play a role in conserving adenine nucleotides. For instance, AMP deaminase deficiency leads to exercise fatigue and poor recovery of the skeletal muscle's adenine nucleotide pool upon termination of intense physical activity (6). Here, we find that inhibiting the PNC with hadacidin prior to ischemia did not enhance the decline of the heart's total adenine nucleotide pool or alter the accumulation of nucleotide degradation products. Only changes in the distribution of adenine nucleotides were observed, which is reflected as a decrease in ATP, ATP:ADP, and adenylate energy charge. The lowering of energy charge under these stress conditions severely limits the heart's ability to perform cellular work.

The preservation of cellular energy status (i.e. ATP:ADP ratio) is one of the previously postulated roles that the PNC plays. This is brought about by the coupling of the adenylate kinase (2 ADP ↔ ATP + AMP) and the AMP deaminase reactions, whereby metabolism of AMP by AMP deaminase shifts the equilibrium of adenylate kinase towards ATP synthesis, thus preserving the ATP:ADP ratio. Data obtained from this study allows us to propose a novel approach in which the PNC preserves energy status. While fumarate reduction at complex II can support ATP production by the ATP synthase, it has been argued persuasively that this mechanism is unlikely to occur both

on theoretical and experimental grounds (2). Inhibition of adenylosuccinate synthetase by hadacidin should reduce the fumarate available for maintenance of anaerobic mitochondrial respiration. As such, the $F_0F_1$ ATP synthase would act in reverse in order to maintain the mitochondrial membrane potential, in the process further depleting ATP. Therefore, reducing the heart's ATP, ATP:ADP, and energy charge during ischemic stress. Thus, our novel proposal is that activity of the PNC prevents the reversal of the $F_0F_1$ ATP synthase in order to maintain the mitochondrial membrane potential, thereby preserving the heart's ATP during ischemic stress. Collectively, we have identified that in addition to glycolysis, the PNC is another crucial mechanism that helps maintain the heart's adenylate energy charge during ischemia. The induction of the PNC by HIF-1α may contribute to the ability of the HIF-1α expressing (6D) hearts to preserve energy charge better than wildtype hearts, at least up to 20 minutes of ischemic stress.

## FIGURES

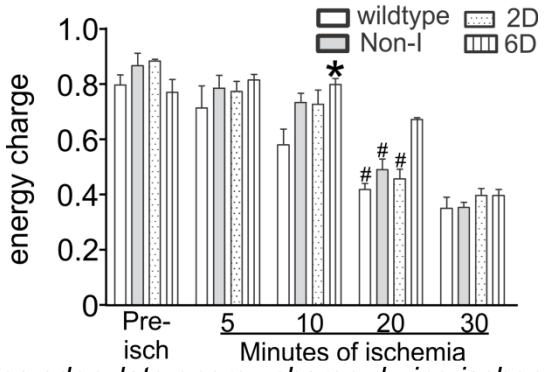


Figure 5.1: *HIF-1α preserves adenylate energy charge during ischemia*. Hearts from wildtype mice and those maintained on doxycycline to suppress the HIF-1α-PPN transgene (Non-I), denied doxycycline for 2 days (2D) or 6 days (6D) to express the HIF-1α-PPN transgene for 2 and 6 days, respectively, were subjected to the indicated durations of *ex vivo* ischemia and nucleotides ATP, ADP, and AMP were measured using HPLC and adenylate energy charge was calculated. Pre-isch = pre-ischemia. * denotes P<0.05 versus wildtype. # denotes P<0.05 versus 6D. n=3-9.

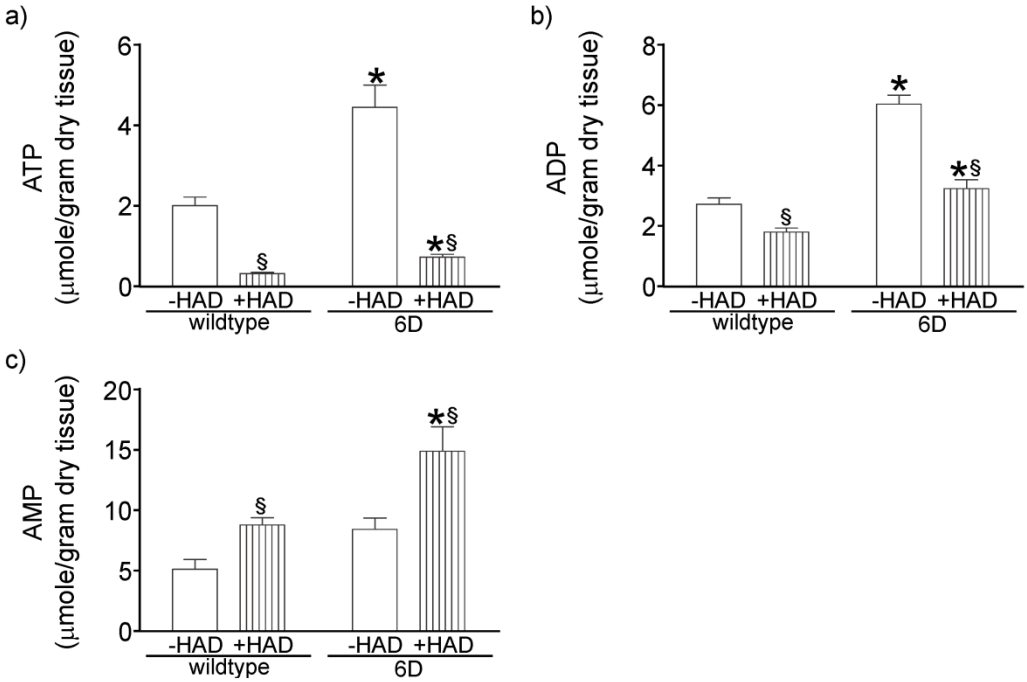


Figure 5.2: *Hadacidin treatment prior to ischemic stress further reduces ATP and ADP but increases AMP accumulation in the heart*. Hearts were obtained from wildtype mice and those denied doxycycline for 6 days (6D) to induce the expression of the HIF-1α-PPN transgene. Hearts were subjected to 30 minutes of *ex vivo* ischemia with or without prior treatment with

PNC inhibitor, hadacidin. After ischemia, nucleotides were extracted from the hearts and measured using HPLC. a) ATP. b) ADP. c) AMP. * P<0.05 versus wildtype of the similar treatment group. § P<0.05 versus hearts without hadacidin treatment. n=5. Note that data from hearts subjected to 30 minutes of ischemia alone were taken from the ischemic time course studies presented in chapter 4.

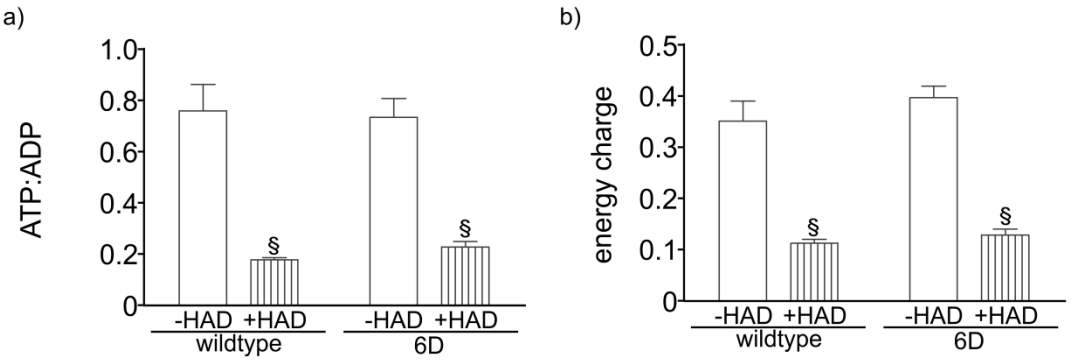


Figure 5.3: *Inhibition of ADSS with hadacidin prior to ischemia drastically decreases the heart's ATP:ADP and energy charge during ischemia*. Wildtype and 6D mouse hearts were subjected to 30 minutes of *ex vivo* ischemia with or without prior treatment with PNC inhibitor, hadacidin. a) ATP:ADP ratio. b) Energy charge. § P<0.05 versus hearts without hadacidin treatment. n=5. Note that data from hearts subjected to 30 minutes of ischemia alone were taken from the ischemic time course studies presented in chapter 4.

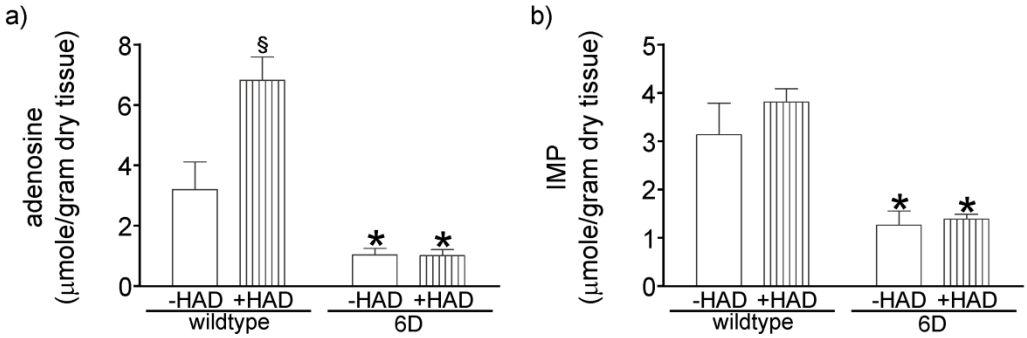


Figure 5.4: *Effects of hadacidin treatment on adenosine and IMP accumulation in ischemic mouse hearts*. Hearts were obtained from wildtype mice as well as those denied doxycycline for 6 days (6D) to induce the expression of the HIF-1α-PPN transgene. Hearts were subjected to 30 minutes of *ex vivo* ischemia with or without prior treatment with PNC inhibitor, hadacidin. After ischemia, nucleotides were extracted from the hearts and measured using HPLC. a) Adenosine. b) IMP. * P<0.05 versus wildtype of similar treatment. § P<0.05 versus hearts without hadacidin treatment. n=5. Data from hearts subjected to 30 minutes of ischemia alone were taken from the ischemic time course studies presented in chapter 4.

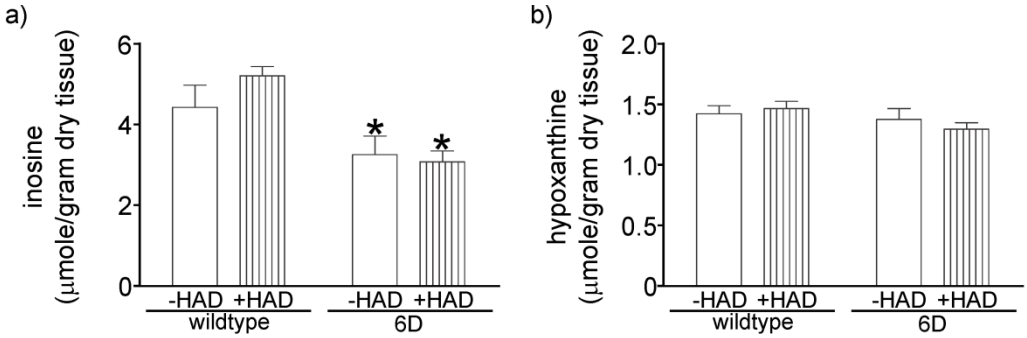


Figure 5.5: *Hadacidin treatment prior to ischemia did not alter inosine or hypoxanthine content in mouse hearts*. Hearts were obtained from wildtype mice and those denied doxycycline for 6 days (6D) to allow expression of the HIF-1α-PPN transgene. Hearts were subjected to 30 minutes of *ex vivo* ischemia with or without prior treatment with PNC inhibitor, hadacidin. After ischemia, nucleotides were extracted from the hearts and measured using HPLC. a) Inosine. b) Hypoxanthine. * $P<0.05$ versus wildtype of similar treatment. n=5. Data from hearts subjected to 30 minutes of ischemia alone were taken from the ischemic time course studies presented in chapter 4.

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# CHAPTER 6

## HIF-1α IN THE HEART: UPREGULATION OF PURINE SALVAGE ENZYME HYPOXANTHINE PHOSPHORIBOSYL TRANSFERASE (HPRT)


## SUMMARY

Ischemia in the heart leads to rapid depletion of the adenine nucleotide pool as well as accumulation of nucleotide degradation products such as hypoxanthine. Hypoxanthine can be utilized by the salvage enzyme, hypoxanthine phosphoribosyl transferase (HPRT) to resynthesize adenine nucleotides. Here we report that HPRT mRNA and protein are induced by hypoxia inducible factor 1α (HIF-1α) in mouse hearts. We have demonstrated that HIF-1α expression leads to increased hypoxanthine accumulation in the ischemic heart. Thus, suggesting that HIF-1α expression provides hearts with a higher capacity to regenerate adenine nucleotides upon recovery from ischemic stress. On the other hand, hypoxanthine can be metabolized by xanthine oxidase upon reperfusion to xanthine. This reaction concomitantly generates a burst of reactive oxygen species (ROS). As a result, the induction of HPRT by HIF-1α may serve the dual purpose of re-incorporating hypoxanthine back into the nucleotide pool and preventing the production of harmful ROS in ischemia-reperfusion.


## INTRODUCTION

During ischemia, the heart's adenine nucleotide pool is readily depleted and nucleobases such as hypoxanthine accumulates (2–4). Hypoxanthine phosphoribosyl transferase (HPRT) is an enzyme that plays a role in nucleotide salvage (Figure 6.1). This enzyme catalyzes the transfer of the 5-phosphoribosyl group on phosphoribosyl pyrophosphate (PRPP) to hypoxanthine, leading to resynthesis of IMP.

Adenylosuccinate synthetase (ADSS), an enzyme of the purine nucleotide cycle (PNC) can utilize IMP to generate AMP, which replenishes the heart's adenine nucleotides. Hypoxanthine can also be degraded to xanthine by xanthine oxidase during reperfusion when $O_2$ is reintroduced to the tissue (Figure 6.1). The reaction catalyzed by xanthine oxidase concomitantly generates a burst of reactive oxygen species in the form of $H_2O_2$. Here, we wanted to determine whether HPRT, an enzyme that can stimulate nucleotide resynthesis and compete with xanthine oxidase for hypoxanthine to potentially reduce ROS is a HIF-1α induced cardioprotective pathway.

## EXPERIMENTAL PROCEDURES

*mRNA expression of HPRT:* The mRNA expression of HPRT in wildtype and HIF-1α Non-I, 2D, and 6D mouse hearts was examined using quantitative PCR. RNA extraction and cDNA synthesis was described under experimental procedures in chapter 3. Quantitative PCR was performed in triplicate for all samples in a 96 well plate format on a CFX96 real-time PCR detection system (BioRad Laboratories, Richmond, CA). 40 cycles were carried out. Each reaction consisted of 50 ng cDNA and 1.5 µl of Quantitect HPRT or transferrin primer from Qiagen in 1x SsoFast Evagreen supermix (BioRad Laboratories). HPRT mRNA expression was normalized to that of reference gene transferrin and results were reported as a percent change in gene expression relative to WT.

*Protein expression of HPRT:* Hearts were obtained from WT, Non-I, 2D, and 6D mice. Protein sample was prepared according to experimental procedures stated in chapter 2. HPRT was probed using a rabbit polyclonal primary antibody diluted to 1

μg/ml in TBS-T (Novus Biologicals, Littleton, CO). The membranes were incubated at 4°C overnight. After incubation in primary antibody, the membranes were washed for 5 minutes in TBS-T (5x) before incubation with 1:2500 goat anti-rabbit HRP conjugated secondary antibody (EMD Millipore, Billerica, MA). Protein bands were detected using the Pierce supersignal chemiluminescence substrate (Thermoscientific) in the G:Box imaging system (Syngene, Frederick, MD). Densitometry of the protein band corresponding to HPRT was obtained using ImageJ (National Institutes of Health, Bethesda, MD) and results were normalized to that of WT.

*Statistical analysis:* Data are expressed as means±SEM. One-way ANOVA was performed followed by SNK post-hoc test using GraphPad Prism 5 (La Jolla, CA).

## RESULTS

Figure 6.2a shows that HIF-1α induces the mRNA of HPRT. In concurrence with the mRNA levels, we show that HIF-1α also induces the protein expression of HPRT (Figure 6.2b).

## DISCUSSION

Here we report that HIF-1α induces the mRNA and protein of nucleotide salvage enzyme hypoxanthine phosphoribosyl transferase (HPRT). We noted in chapter 4 that HIF-1α expression enhances the accumulation of hypoxanthine during ischemia. Hypoxanthine can either be a good or harmful metabolite. It can be utilized by HPRT to resynthesize adenine nucleotides. However, it can also be converted by xanthine oxidase to xanthine when oxygen is reintroduced during reperfusion. The metabolism of hypoxanthine by xanthine oxidase leads to a burst of ROS production. It has been

demonstrated that oxypurinol, a xanthine oxidase inhibitor reduces ROS and improves cardiac function after ischemia-reperfusion stress (5). Thus, the induction of HPRT by HIF-1α may divert hypoxanthine away from xanthine oxidase metabolism, leading to attenuation of ROS. Taken together, the induction of HPRT by HIF-1α may enhance the re-assimilation hypoxanthine back into the adenine nucleotide pool and prevent production of harmful ROS during ischemia-reperfusion.

## FIGURES

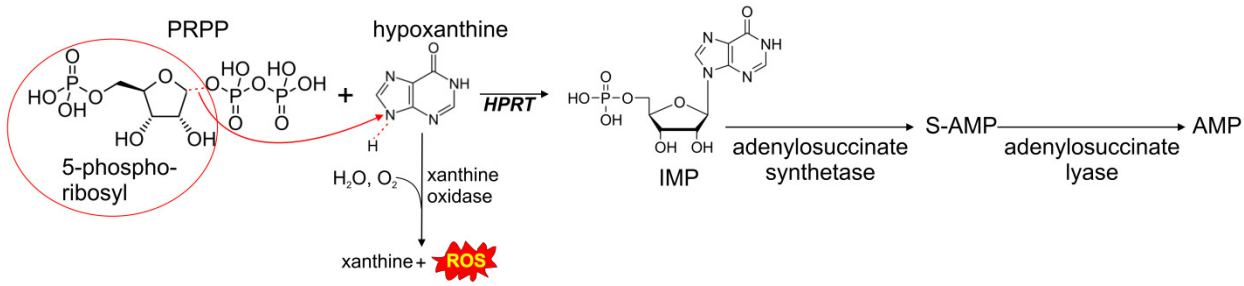


Figure 6.1: *Hypoxanthine phosphoribosyl transferase (HPRT) reaction mechanism.* HPRT catalyzes the transfer of the 5-phosphoribosyl group on phosphoribosyl pyrophosphate (PRPP) to hypoxanthine to resynthesize IMP. Adenylosuccinate synthetase, an enzyme of the purine nucleotide cycle (PNC) can utilize IMP to regenerate AMP, which replenishes the heart's adenine nucleotides. The availability of $O_2$ during reperfusion allows xanthine oxidase to convert hypoxanthine to xanthine, a reaction that also synthesizes a burst of ROS. Therefore, HPRT induction in HIF-1α expressing hearts may also function to minimize ROS production during ischemia-reperfusion insult.

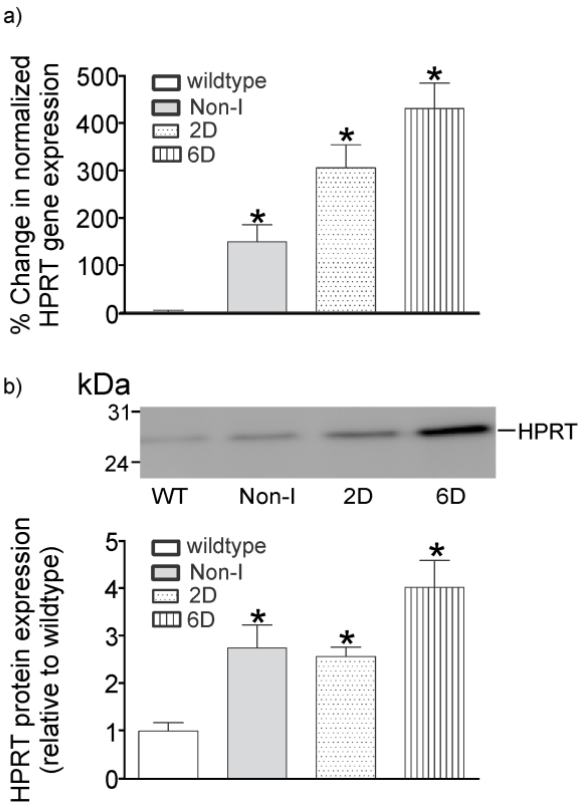


Figure 6.2: *HIF-1α upregulates the mRNA and protein of hypoxanthine phosphoribosyl transferase (HPRT) in mouse hearts.* HPRT mRNA and protein was examined in wildtype (WT)

mouse hearts and those where the HIF-1α-PPN transgene was suppressed (Non-I), expressed for 2 days (2D), or expressed for 6 days (6D). a) HPRT gene expression in mouse heart homogenates was examined using qPCR and normalized to reference gene transferrin. Results are expressed as the % change in gene expression relative to WT (n=6). b) Representative Western blot showing HPRT protein expression in mouse heart homogenates (top) and quantification of HPRT protein levels in mouse heart homogenates (n=4-7). * $P<0.05$ versus WT.

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# CHAPTER 7

## SUMMARY & FUTURE DIRECTIONS

These studies showed that HIF-1α expression allows for strong protection of the heart during ischemic stress. We observed that ATP preservation during ischemia in our HIF-1α non-induced hearts (Non-I) was not better compared to wildtype hearts, yet the Non-I hearts were protected to the same extent as hearts that have been expressing HIF-1α for 2 and 6 days. These results suggest that the ability to preserve ATP is not necessarily required to protect the heart from ischemic stress. Evidence showing that many of the protective mechanisms that lead to ischemic tolerance involve the protection of the mitochondria is beginning to emerge.

Here we showed that myocytes isolated from HIF-1α Non-I, 2D, and 6D mouse hearts were able to maintain mitochondrial membrane potential better than myocytes obtained from wildtype mouse hearts. Previous studies by our group demonstrated that the HIF-1α signaling axis induces the ability for the mitochondria to use purine nucleotide cycle derived fumarate as a terminal electron acceptor in place of $O_2$ to sustain anaerobic mitochondrial respiration. In chapter 3 we showed that AMP deaminase 2, the entry point to the PNC is upregulated in extracts obtained from 6 day HIF-1α expressing hearts as compared to wildtype. Here we have obtained evidence indicating that the protective effects provided by the PNC extends beyond the capability to maintain mitochondrial membrane potential during ischemia.

Treatment of hearts with hadacidin, inhibitor of the PNC at the step of adenylosuccinate synthetase followed by ischemia led to a decline in ATP, ATP:ADP,

and adenylate energy charge. Inhibition of ADSS with hadacidin should reduce the amount of fumarate available for anaerobic mitochondrial respiration. This will result in the reversal of the $F_0F_1$ ATP synthase in order to maintain the inner membrane potential of the mitochondria during ischemic stress, which will further deplete ATP. Thus, in addition to sustaining the mitochondrial membrane potential, the PNC also exerts ATP sparing effects during ischemia.

A significant finding in these studies is that HIF-1α limited the adenosine that accumulates in the ischemic heart. While consistent with the shunting of AMP metabolism towards the PNC rather than the adenosine producing 5'-nucleotidase pathway, we also find that HIF-1α upregulates adenosine deaminase. Adenosine deaminase further degrades adenosine to inosine, thereby contributing to the lower adenosine levels that we have observed in the HIF-1α expressing hearts. Also, we were surprised to see that IMP accumulation remained lower in HIF-1α expressing hearts as compared to wildtype mouse hearts during ischemia, even though HIF-1α upregulates AMP deaminase. Manfredi and Holmes proposed that some mechanism prevents the accumulation of IMP in the ischemic heart. Taken together, it is likely that a multitude of factors determine the instantaneous levels of IMP during ischemia. Thus, we would like to obtain precise quantification of flux through the PNC in the future.

The observation that HIF-1α limits adenosine accumulation in the ischemic heart contradicts the protective function ascribed to adenosine, particularly from the perspective of cardioprotection provided by ischemic preconditioning. However, recent evidence has pointed out that chronic adenosine exposure can lead to toxicity. Future

investigations will establish whether HIF-1α can help the ischemic heart avoid adenosine toxicity.

Our studies have also led to the finding that HIF-1α induces the expression of the nucleotide salvage enzyme, hypoxanthine phosphoribosyl transferase (HPRT). HPRT catalyzes a reaction that converts hypoxanthine back to IMP. IMP is then converted back to AMP via two reaction steps: 1) IMP to S-AMP by adenylosuccinate synthetase; 2) S-AMP to AMP by adenylosuccinate lyase. HPRT may also act in reverse to metabolize IMP to hypoxanthine. While highly speculative, the increased accumulation of hypoxanthine during ischemia in HIF-1α expressing versus wildtype hearts maybe consistent with the faster rate of IMP metabolism by HPRT. This provides a plausible mechanism that could lead to lower IMP levels in our HIF-1α expressing hearts as compared to wildtype during ischemia. In addition to its role in adenine nucleotide salvage, HPRT may also limit the amount of harmful reactive oxygen species (ROS) generated during reperfusion. Future studies will establish that the induction of HPRT in HIF-1α expressing hearts allows for better recovery of nucleotides and limits ROS-induced injury during reperfusion.

Taken together, we found that HIF-1α expression in the heart provides strong protection during ischemic stress. Further, we identified that HIF-1α induces several metabolic pathways that could contribute to cardioprotection. Future work will aim to quantify the flux through these metabolic pathways and to further elucidate the role of these pathways in cardioprotection during ischemia.

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